UPTAKE OF AMINO ACIDS BY THE CYSTICERCOID OF HYMENOLEPIS DIMINUTA (CESTODA)

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TITLE "UPTAKE OF AMINO ACIDS BY THE CYSTICERCOID OF HYMENOLEPIS DIMINUTA (CESTODA)"

I certify:

(i) That the greater portion of the work submitted in the above thesis has been done subsequent to my registration for the degree of Ph.D.

(ii) That the above thesis is my own account of my own research.

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ABSTRACT

The cysticercoid of Hymenolepis diminuta was found to absorb, by a process of active mediated transport, the synthetic amino acids cycloleucine (cyclo), α-aminoisobutyric acid (α-AIB), and N-methylaminoisobutyric acid (N-MeAIB) and the naturally-occuring amino acids L-alanine (L-ala), L-leucine (L-leu), L-proline (L-pro) and L-phenylalanine (L-phe). All of these compounds were accumulated within the cysticercoid and the four natural amino acids were incorporated into protein. The K_t (Transport Constant) describing the uptake of cycloleucine remained unchanged irrespective of the age or developmental stage of the cysticercoid, but the V_{max} (Maximum velocity of uptake) declined as the cysticercoid aged, and also, at a fixed parasite age, as the infection density within the beetle host rose. The uptake of cycloleucine could be inhibited by a wide variety of neutral L-amino acids and their analogues, the degree of inhibition being dependent on their structure. Sugars, betaine, sodium acetate, basic and acidic amino acids did not interact with the cycloleucine uptake loci. D-methionine was a far less effective inhibitor than L-methionine. In every case where inhibition occured, it was found to be of a competitive nature. The K_t values describing the uptake of all seven amino acids studied, except L-pro, were very similar to those obtained with the adult worm. However, the L-pro value was much higher than the equivalent adult figure, and may be related to the high proportion (68%) of this compound in Tenebrio haemolymph. By examining the reciprocal inhibitory activities of all the amino acids studied (except N-MeAIB),

it was found that the entered the cysticercoid through three distinct transport loci. The transport of amino acids in adult *H. diminuta* and cysticercoids is compared.

CONTENTS

1. INTRODUCTION

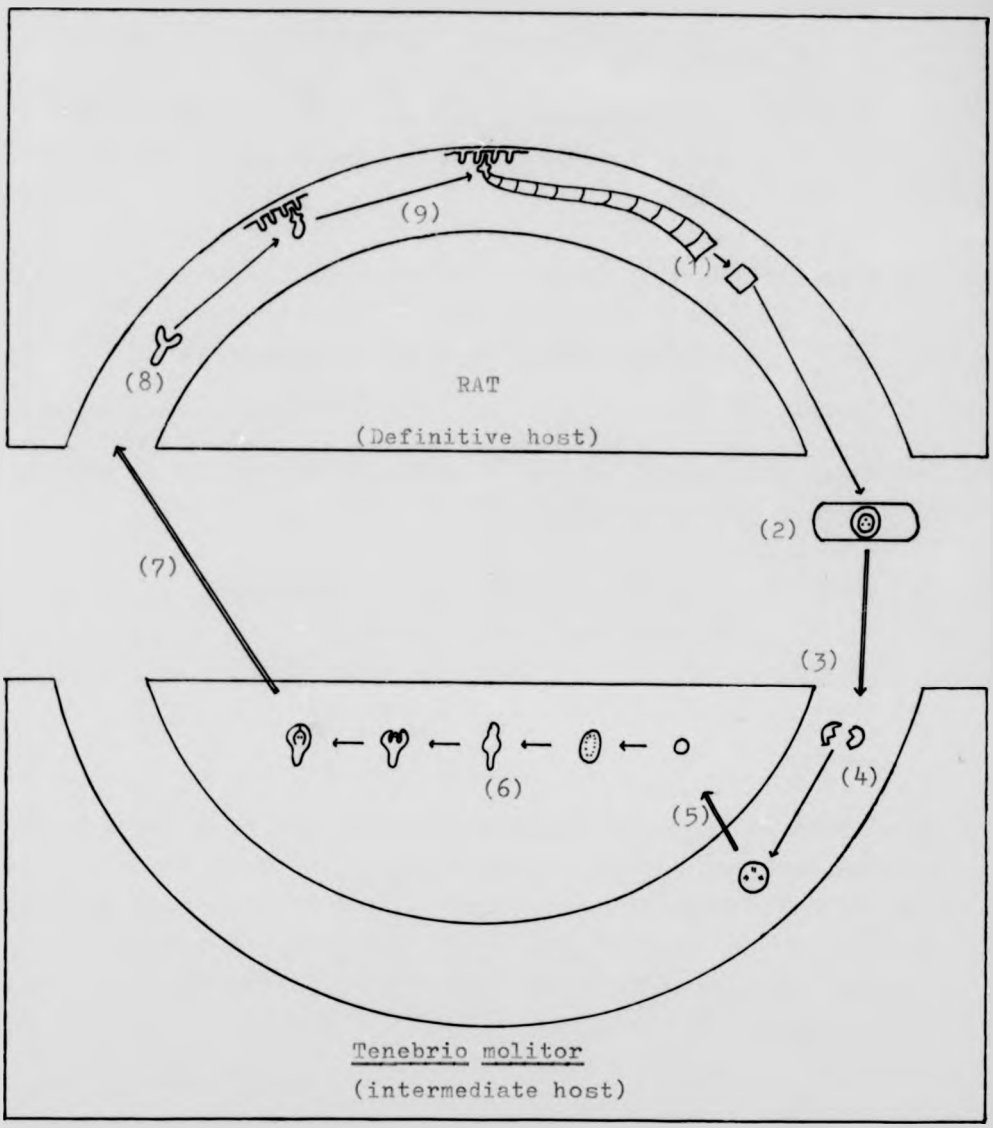
A parasite may be defined as "... an organism which is in continuous, intimate association with another organism, the host, on which it is metabolically dependent to a greater or lesser degree" (Smyth, 1976). This metabolic dependence may be nutritional and may also involve other factors, for example, the temperature stimulus provided by the final host for the development of some pseudophyllidean tapeworms (e.g. Schistocephalus solidus, Smyth, 1954, 1959). As tapeworms lack a digestive tract, that portion of the tapeworm in intimate association with the host organism must be of the greatest importance in nutrition. This region has been termed the 'host-parasite interface' by Read, Rothman & Simmons (1963) and defined by them as "... a region of chemical juxtaposition of regulatory mechanisms of both host and parasite". Read et al. interrreted the interface as being the external surface membrane of the worm, but it is now considered that the interface consists not only of this membrane, but the glycocalyx, an 'unstirred' region within, or closely associated with the glycocalyx and adsorbed host components (Pappas & Read, 1975; Pappas, 1983a, b).

Much is known of the properties of this interface in some helminths, but surprisingly little thought has been directed towards its functioning in a changing environment. The life cycle of a parasite invariably involves the utilisation of several hosts, and possibly a number of free-living stages. At each of these phases of development the parasite will be

exposed to differing physico-chemical environments to which it must adapt to survive. This adaption may take the form of changes in the physiology, biochemistry and structure of the parasite. This thesis examines the nutritional physiology of the metacestode of Hymenolepis diminuta, which inhabits the haemocoel of a number of insects, and a comparison will be made with the nutritional physiology of the adult stage which is found in the small intestine of the laboratory rat (see Figure 1.1. for the lifecycle of H.diminuta). Possible changes in the properties of the interface as the parasite moves from one environment to the other will be examined by comparing the characteristics of the amino acid transport systems at both stages of the life cycle. However, the metacestode (hereafter termed the cysticercoid) goes through a series of developmental stages in its intermediate host, Tenebrio molitor, during which its gross morphology and structure changes considerably (see Figure 1.2.), and it is possible that the properties of the interface may change with time as the cysticercoid develops. The aims of this thesis are thus twofold:-

a.) Bearing in mind the widely different environments experienced by the adult and cysticercoid stages of H.diminuta, to examine whether there are any differences in the characteristics of the amino acid transport systems possessed by these two stages.

b.) To determine if the age-dependent morphological changes which occur during cysticercoid development are associated with changes in the pattern of nutrition.



Life cycle of Hymenolepis diminuta (original). (1)-gravid proglottides are shed by the adult worm, and the eggs pass out in faecal pellets (2). On ingestion by a suitable arthropod (3), the oncosphere is liberated from the egg in the beetle gut (4) and penetrates the gut wall (5) to enter the haemocoel where it develops to an infective cysticercoid in 9 days at 26°C (6). On the ingestion of the beetle by a rat, the cysticercoid is liberated and excysts in the small intestine (7, 8). The liberated metacestode attaches itself to the mucosa of the gut and develops to an egg-laying adult worm within 12 days (9).

Figure 1.1.

Legends to Figure 1.2.

(a) Stage I. Solid sphere of cells and paired oncosphere hooks. (Drawing taken from photograph).

(b) Stage II (Late). Elongation and extension of cavity (forms during Late Stage I). (Drawing taken from photograph).

(c) Stage III (Early). Three body divisions, extension of cavity with surrounding fibres. Drawing taken from original drawing of Voge & Heyneman, as are figures d-f.

(d) Stage III (Late). Showing outline of suckers and indication of rostellum.

(e) Stage IV. Recently withdrawn scolex surrounded by thin layer of withdrawn "neck" tissue.

(f) Stage V. Mature infective cysticercoid.

Developing in Tribolium confusum maintained at 30°C, Stage I is reached after an average time of 36 hours, Stage II after 56 hours, III after 84 hours, IV after 132 hours and the infective Stage V phase after 180 hours (Voge & Heyneman, 1957). When reared in Tenebrio molitor, Stage V is reached in approximately 9 days at 26°C (personal observation).

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Figure 1.2. Developmental stages of the H.diminuta cysticercoid (redrawn from Voge and Heyneman, 1957). For descriptions of stages, see adjacent page. Scale bar applies to figures c-f only.

For an understanding of the role of the interface in the parasite-host relationship one must consider both its structure and function. The following pages briefly review these factors, with particular reference to H.diminuta.

Until the 1960's microscopical examination of the outer regions of tapeworms revealed them to be relatively homogenous and this zone was widely referred to as a cuticle. However, electron microscopists revealed that this 'cuticle' consisted of a distal syncytium, bounded at its apical and basal surfaces by membranes and continuous at its basal surface with nucleated cell bodies. The skin of the tapeworm is now termed the 'tegument', the syncytial region the 'distal cytoplasm' and the underlying cell bodies 'tegumentary cytons' which connect with the distal cytoplasm via 'internuncial canals'. The apical surface of the tegument is typically thrown into digitiform structures which may be either 'microtriches' or 'microvilli'. The majority of the former are distinguished from the latter by the possession of a terminal keratinous spike, set off from the shaft of the microthrix by a multilaminate baseplate. The structure of the tegument of an adult tapeworm is shown diagrammatically in Figure 1.3., and is discussed in further detail in the reviews of Lumsden (1975), Lumsden & Murphy (1980) and Lumsden & Hildreth (1983)(for tapeworms in general) and Lumsden & Specian (1980)(for adult H.diminuta in particular). Due to its similarity to the arrangement of the absorptive surface of the mammalian small intestine, the tapeworm surface is often referred to as a 'brush border'. Possible functions of the brush

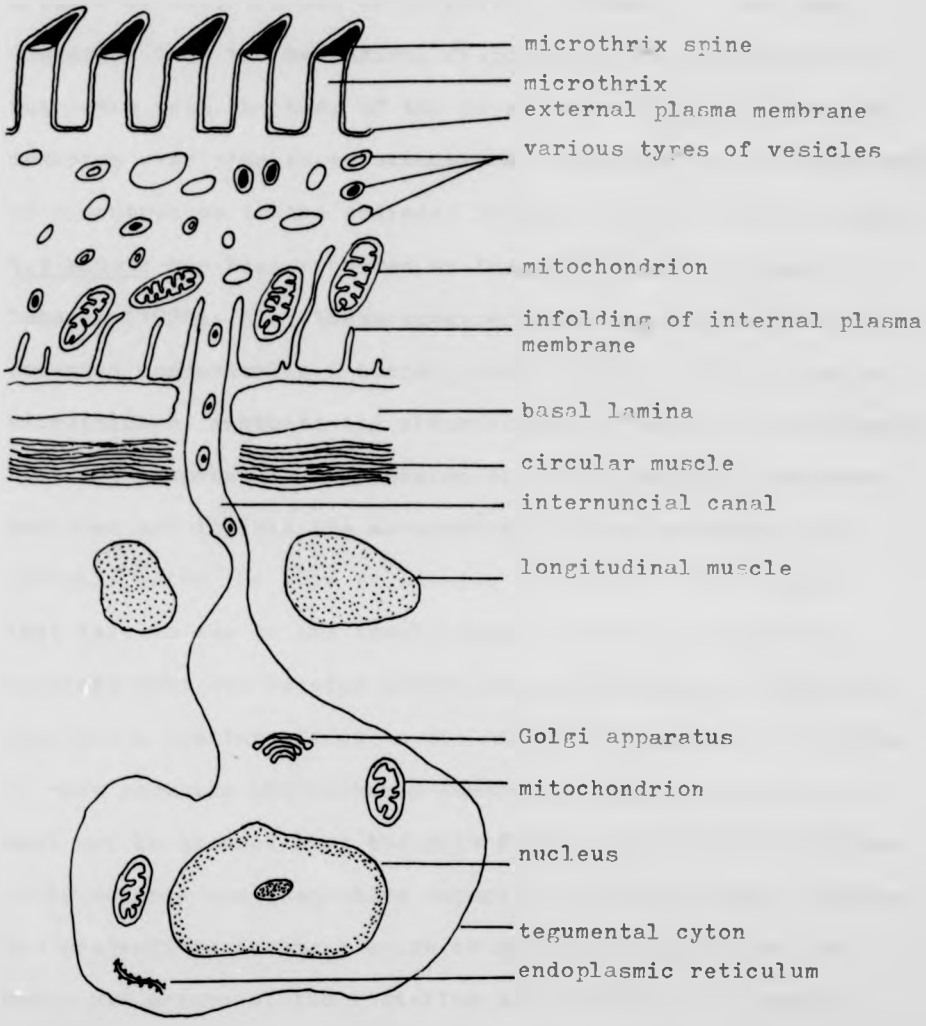


Figure 1.3. Generalised diagram of the cestode tegument (after Smyth, 1972).

border will be discussed shortly but one important role would appear to be to amplify the free surface of the tegument. An increase of 2.6 over a plane surface due to the presence of this layer has been calculated for adult H.diminuta by Berger & Mettrick (1971). Due to the increased surface area that a layer of microtriches or microvilli affords, it has been suggested that the mechanisms responsible for the transport of nutrients into the body of the tapeworm are located within the membrane overlying these structures. Evidence for the involvment of microtriches in the mediated transport of nutrients by adult H.diminuta has been provided by the studies of McCracken & Lumsden (1975a, b). These workers have observed that the compound Concanavalin A (ConA), which binds to the surface of microtriches, inhibits the absorption of a variety of nutrients that are absorbed by a mechanism of active mediated transport, but does not inhibit the absorption of those compounds that normally enter the worm by passive diffusion. They suggest that this is due to the immobilisation by ConA of specific carriers that are located within the microtriches. Phlorizin also has a similar effect on the mediated transport of glucose by this parasite (McCracken & Lumsden, 1975a). However, it must not be assumed that the sole function of the microtriches is to enhance the absorptive capacity of the tapeworm. Because the projections assume a spine or hook-like appearance (the hooks and spines of the rostellum are thought to be highly developed microtriches (Mount, 1970)) it has been suggested that they contribute to adhesion (either in migration or maintaining position against the peristalsic forces of the intestine) and

in addition may serve to ward off impactment by host cells (Lumsden & Hildreth, 1983).

Modifications of the external body surface to resemble an absorptive layer appear at an early stage of tapeworm development. The oncosphere of H.diminuta bears a syncytial epithelium bounded externally by an apical plasma membrane that is thrown into many filamentous projections forming a loose skirt around the oncosphere (Lethbridge & Gijsbers, 1974; Pence, 1970; Rybicka, 1973). The ultrastructure of the stage V cysticercoid has been extensively reviewed by Ubelaker (1980). Figure 1.4. is a diagrammatic representation of the outer wall of the capsule of the cysticercoid. In its general structure it resembles the tegument of the adult worm, with the exception that the surface projections are microvilli and not microtriches. It is clear that the sparse layer of microvilli do not afford so great an increase in surface area as the densely packed microthrix layer of the adult, and it has been suggested by Smyth (1972) that this difference may be related to the greater nutritive requirements of the adult worm. Similar differences in the density and structure of larval and adult absorptive layers are apparent in Dirhyllobothrium latum (Brøten, 1968a, b) and D. (Spirometra) erinacei (Yamane, 1968). Elsewhere on the cysticercoid of H.diminuta, the presumptive scolex surface closely resembles that of the adult worm, bearing well-formed microtriches, suckers and a rostellar region (Ubelaker, 1980; Richards & Arme, 1984)

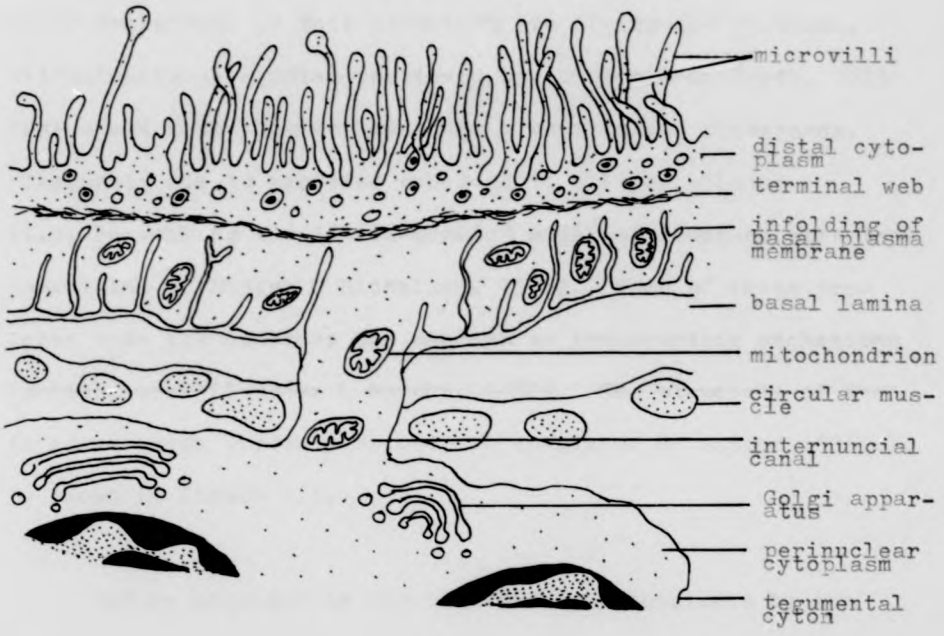


Figure 1.4. H.diminuta - L.S. outer wall of capsule of stage V cysticercoid (after Ubelaker, Cooper & Allison, 1970a).

Overlying the brush border membrane is the glycocalyx. Histochemical analyses and ion-binding studies have suggested that the glycocalyx is composed of carbohydrates (probably glycoconjugates) and is strongly anionic, thus bearing a net electronegative charge. The composition and functions of the glycocalyx have recently been reviewed by Pappas (1983a, b). Using the technique of Oaks, Knowles & Cain(1977), Knowles & Oaks (1979) have prepared a pure sample of brush border membrane from adult H.diminuta, analyses of which suggest that the major components of this structure are lipids and proteins. Ultrastructural studies (reviewed by Lumsden & Hildreth, 1983) have shown that this membrane has a particulate appearance, presumably due to proteins embedded in a lipid bilayer (i.e. resembling the 'fluid-mosaic' model of membrane structure postulated by Singer & Nicholson, 1972). Some of these proteins span the membrane and may act as transporting mechanisms (permeophores)(Lumsden & Murphy, 1980). The structure of the tapeworm brush border membrane, as envisaged by Pappas (1983a), is shown in Figure 1.5.

Before considering how the uptake of nutrients by the tapeworm is measured and described, and the factors that may affect this, it is worthwhile summarising the methods whereby a cell may obtain such nutrients and whether these methods are used by the tapeworm:-

a.) Through pores or other special channels such as cell-cell junctions. Ultrastructural studies have not provided

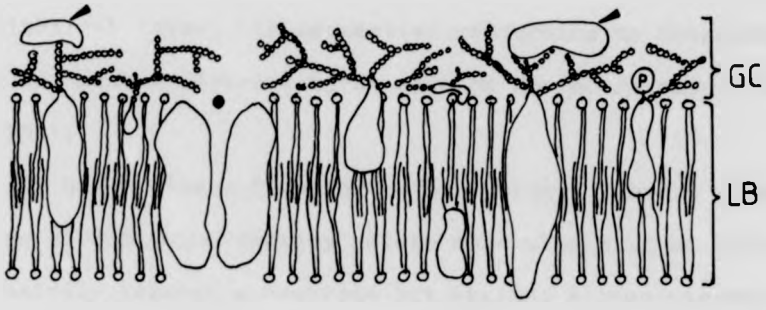


Figure 1.5. Idealised representation of the brush border membrane of H.diminuta based on the fluid-mosaic model. The lipid bilayer (LB) contains several glycoproteins, the carbohydrate portions of which (small circles) form the glycocalyx (GC). P is a phosphoprotein. A carrier, the external opening of which is shown by a solid circle, is depicted at the left. Two host proteins, such as enzymes or immunoglobulins, adsorbed to the glycocalyx, are also shown (arrows) (after Pappas, 1983a).

any evidence for the existence of such structures in the tegument of adult H.diminuta, and the membrane transport studies of Podesta and his co-workers (summarised in Podesta, 1980, 1982) have shown that the syncytial tegument behaves like a 'tight' epithelial layer, its properties conforming to those predicted by the double membrane hypothesis of Koefed-Johnsen & Ussing (1958).

b.) By 'mass flow' or solvent drag. This is a specialised form of diffusion whereby solute molecules will be transported passively through a membrane but against a concentration gradient where there is a large solvent flow. In general, the mediated transport of water against a concentration gradient can be explained by a primary transport of a solute, usually an ion such as Na^+, with water following passively due to osmotic forces. For example, the reabsorption of water from the cockroach rectum has been found to be primarily dependent on the active transport of Na^+ into the rectal pads. This increases the concentration of the fluids in this area to such an extent that they become more concentrated than those in the rectal lumen, but less concentrated than the blood. Water will thus move from the rectal lumen into the rectal pads (Wall, Oschman & Schmidt-Nielsen, 1970). Adult H.diminuta is also capable of absorbing ions and water from its ambient medium (Podesta & Mettrick, 1975, 1976), and there is some evidence that the fluxes of these compounds across the tegument are linked with the transport of glucose (Podesta & Mettrick, 1976). At the present time, however, there is insufficient evidence to conclusively prove that glucose enters H.diminuta by mass flow.

c.) By endocytosis. Hopkins, Law & Threadgold (1978) have shown that the plerocercoid of S.solidus is capable of endocytosing the electron-dense tracers horseradish peroxidase, ruthenium red and lanthanum nitrate. These compounds were transported across the tegument and exocytosed from the tegumental base within 5 minutes, while in adult S.solidus and the plerocercoid of Ligula intestinalis ruthenium red was endocytosed, transported transtegumentally and exocytosed within 6 minutes (Threadgold & Hopkins, 1981). Threadgold & Dunn (1983) have recently provided evidence that endocytosis also occurs in the tegument of the cysticercus of Taenia crassiceps. It is only in these three examples that endocytosis has been demonstrated uncontroversially. Although Rothman (1967) reported that adult H.diminuta was permeable to ferritin, colloidal thorium dioxide and carbon, these observations could not be confirmed in a later study (Lumsden, Threadgold, Oaks & Arme, 1970). Substances of host origin have been identified in the 'bladder' fluids of several larval cyclophyllidean cestodes, and experimentally some of these species have been found to absorb various other compounds (see Table 1.1.). However, the mechanisms by which these substances enter the parasites is unknown. The role of endocytosis where it does occur is obscure. A nutritional function is an obvious explanation, but the presence of a well developed microthrix layer in all the cited examples relegates endocytosis to at least an ancillary role in this particular function. Threadgold & Hopkins (1981) have suggested that endocytosis in the plerocercoids of I.intestinalis and S.solidus may be involved in the parasites' response to the

immune attack of the host. The presence of abundant vesicles, pinosomes and lysosome-like bodies in the tegument of these two parasites indicates a rapid turnover of surface membrane components. Indeed it is known that the adult H.diminuta replaces its glycocalyx every 6-8 hours (Oaks & Lumsden, 1971). Such a process could have survival value to the plerocercoids, as any antigen-antibody complexes would be rapidly internalised, catabolised and recycled through the parasites' lysosomal system. A flaw in this hypothesis is the high degree of host specificity shown by the plerocercoid of S.solidus which will only develop in the body cavity of Gasterosteus aculeatus, being unable to resist the immune attack of other fish (Orr, Hopkins & Charles, 1969). Possibly other fish antibodies do not induce this protective response.

d.) By passive diffusion. This form of transport can be distiguished experimentally from carrier mediated transport by the observed linear rate of uptake with respect to increasing solute concentration (see line A, Figure 1.6.1.) and the inability of the cell to accumulate the solute against a concentration gradient. Additionally, the rate of passive diffusion is not affected by competition from structurally-similar compounds or dependent on metabolic energy sources. Diffusion has been demonstrated in the transport of some metabolites across tapeworm brush border membranes but is usually apparent at higher solute concentrations and may not be of physiological significance in every case (see Pappas & Read, 1975). In adult H.diminuta diffusion accounts for a proportion of the transport of proline (Kilejian, 1966a), histidine (Woodward & Read, 1969),

Table 1.1. Permeability of cyclophyllidean tapeworms to macromolecules

Species	Macromolecule	References
Echinococcus granulosus (Hydatid cyst)	HP[a]-sheep	Norman, Kagan & Chordi (1964); Chordi & Kagan (1965); Coltorti & Varela-Diaz (1972)
	HP-rats & mice	Hustead & Williams (1977a)
	HP-humans & gerbils	Coltorti & Varela-Diaz (1972)
	Peroxidase & IgG	Coltorti & Varela-Diaz (1974, 1975)
Taenia crassiceps (Cysticercus)	HP-mice	Hustead & Williams (1977a)
	^{14}C-*Chlorella* protein	Esch & Kuhn (1971)
	BSA[b], IgG & RN[c]	Hustead & Williams (1977a, b)
Taenia taeniaeformis	HP-rats	Hustead & Williams (1977a)
	BSA, IgG & RN	Hustead & Williams (1977a, b)

a = host protein, b = bovine serum albumin, c = ribonuclease

phenylalanine, tryptophan and tyrosine (Pappas & Gamble, 1980), glycerol (Pittman & Fisher, 1972; Uglem, Pappas & Read, 1974), uracil, adenine and hypoxanthine (MacInnis, Fisher & Read, 1965), acetate (Arme & Read, 1968), palmitate (Chappell, Arme & Read, 1969) and several water-soluble vitamins (see Pappas & Read, 1975).

e.) By carrier-mediated transport. Experimentally this process exhibits saturation kinetics (see line B, Figure 1.6.1.). It has been suggested that mediated transport involves the reversible binding of a substance to a specific site at which it undergoes transport. The rate of binding, transport across the membrane by a mobile carrier and release of the substance on the inside of the membrane set the upper limit to the rate of transport, just as the rate of formation and breakdown of an enzyme-substrate complex can limit the rate of an enzyme catalyzed reaction. Membrane transport thus shares many features with enzyme kinetics, and the terminology employed for the latter is used to describe the former, except that in transport the 'catalytic'event is the transmembrane movement of a solute, not the production of a defined molecule. Further features of mediated transport are the exhibition of specificity, and often stereospecificity.for the substance transported, the inhibition of uptake by structurally-similar compounds and a dependence on metabolic energy. Mediated transport, or strictly, mediated influx, is an important feature of the transport of most compounds by adult H.diminuta as well as the absorption of α-aminoisobutyric acid (α-AIB), glucose and sodium acetate by the cysticercoid of this parasite (Arme & Coates, 1971; Arme & Coates, 1973; Arme, Middleton & Scott, 1973). Mediated transport

can be separated into two types, facilitated diffusion and active transport, by a demonstration in the latter case of the accumulation of the test compound against a concentration gradient. Active transport of amino acids by adult H.diminuta has been implicated in the transport of methionine (Read et al., 1963), proline (Kilejian, 1966b), histidine (Woodward & Read, 1969), cycloleucine and α-AIB (Harris & Read, 1968) and phenylalanine, tyrosine and tryptophan (Pappas & Gamble, 1980). The cysticercoid of H.diminuta also accumulates α-AIB (Arme & Coates, 1973). Facilitated diffusion has been implicated in the absorption of glycerol by Taenia taeniaeformis metacestodes (Von Brand, Churchill & Higgins, 1966) and of sodium acetate by adult H.diminuta (Arme & Read, 1968). The efflux of solutes out of the tapeworm will not be considered at this point but will be referred to at the appropriate places later in this study.

f.) By the mediation of enzymes. The brush border membrane of certain cestode species bears surface enzymes of both parasite and host origin, and can thus be considered as a digestive-absorptive surface (Arme & Read, 1970). H.diminuta also interacts with trypsin, α- and β-chymotrypsin and lipase in vitro (Pappas & Read, 1972a, b; Ruff & Read, 1973). However, these enzymes are either irreversibly inactivated (trypsin and the chymotypsins) or inhibited (lipase). Protease inactivation may be concerned with protecting the worm from the proteolytic activity of the host, although the significance of lipase inhibition remains obscure. Thus the brush border membrane may also function as a protective surface. There is some evidence to suggest that certain surface enzymes of tapeworms are located

in close spatial proximity to certain transport systems of these parasites. Dike & Read (1971) showed that adult H.diminuta was capable of hydrolysing glucose-6-phosphate and that the freed glucose moieties were preferentially absorbed over free glucose in the medium. This data suggests a close proximity of the phosphohydrolases to the glucose transport systems but the separate identity of the two sites can be shown by the fact that if the functioning of one is inhibited the other continues to operate normally (Dike & Read, 1971). Pappas & Read (1974) investigated the interactions of nucleotide hydrolysis and nucleoside transport in H.diminuta, and concluded that the nucleoside transport system and phosphohydrolases of this worm were located in such a way that the products of adenosine triphosphate hydrolysis interacted with the nucleoside transport system rather than diffusing into the ambient medium. In both of these cases, the arrangement of enzymes to transport systems would result in a 'kinetic advantage' for absorption of hydrolysis products. This might be significant in allowing the parasite to compete more effectively with the host for necessary nutrients and metabolic intermediates (Pappas & Read, 1975). Three enzymes of the γ-glutamyl cycle, a catalytic chain of events resulting in the transport of an amino acid, have been identified in homogenates of Moniezia expansa (Kurelec & Rijavec, 1976). However, it is not known whether this cycle is functional in the nutrition of M.expansa and whether it exists in other tapeworms.

The methods by which the transfer of nutrients across biological membranes are first distinguished and then quantified are, as noted above, derived from enzyme kinetics, with the distinctions that a mobile carrier molecule (C), and a molecule of the compound to be transported (S) take the place of the enzyme (E) and the substrate (S) respectively, and the catalytic event is not the formation of a product, but the translocation of a molecule from one phase (S_1) to another (S_2). Mediated transport can thus be described by equation (1):-

$$S_1 + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} SC \xrightarrow{k_3} C + S_2 \qquad -(1)$$

Experimentally the process is allowed to continue for a time period insufficient to permit efflux of the test compound, and thus only the unidirectional flux, v, which proceeds at a rate k_3 is measured and given by equation (2):-

$$v = k_3[SC] \qquad -(2)$$

If one further assumes that the rate of SC formation is constant, the rate of transport can be described by equation (3):-

$$v = \frac{V_{max} \cdot [S]}{K_t + [S]} \qquad -(3)$$

Where V_{max} is the highest rate, under fixed conditions, that a substance can be absorbed by a given quantity of biomass (and is indirectly a measure of the number of transporting systems per unit biomass), and K_t, the transport constant, gives a measure of the affinity of these transport systems for the compound

studied. The full derivation of these constants is provided in Appendix 2. K_t and V_{max} can be calculated in a variety of ways, but most involve the transformation of kinetic data to a linear format, the most commonly employed transformation being the double reciprocal plot of Lineweaver & Burk (1934) (see Figure 1.6.2.). A consideration of the advantages and disadvantages of linear methods such as these are fully discussed in Section 4.2.2.

Experimentally, the kinetic analysis of uptake commences by determining an 'initial rate period'. This is defined as a time period over which the uptake of the test compound remains linear with respect to time, and is presumed to be an indicator of the unidirectional influx of the test compound. Any deviation from linearity after this time period may be due to a variety of causes, for example, changes in pH, $[O_2]$, $[CO_2]$, cell death, substrate depletion or substrate efflux. Once determined, the next step is to examine the uptake rates at this fixed time period over a range of test compound concentrations ([S]), and plot the results as uptake, v, against [S] (a Michaelis-Menten plot. In most cases three possible results occur, and these are shown in Figure 1.6.1. Line A represents passive diffusion, B mediated transport and C_1 a combination of both processes. Line C_1 can be transformed mathematically to give line C_2, indicating the proportion of uptake that can be attributed to mediated transport. It must be stressed however that the form of the line in a Michaelis-Menten plot does not conclusively prove that the uptake of the test compound occurs by one or

Figure 1.6. Graphical methods of kinetic analysis (see text for details)

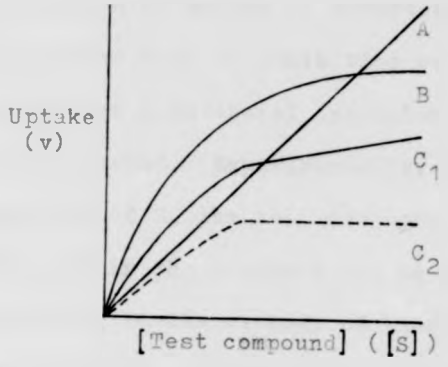


Figure 1.6.1. Possible outcomes of kinetic experiments.

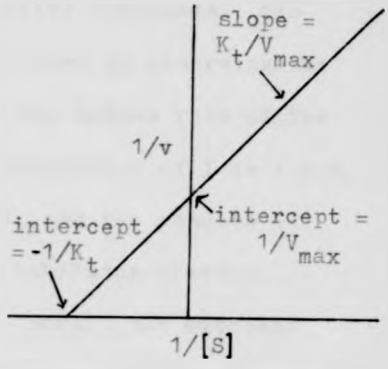


Figure 1.6.2. Calculation of K_t and V_{max} by method of Lineweaver & Burk.

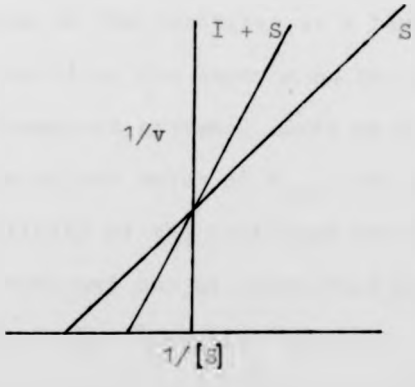


Figure 1.6.3. Competitive inhibition. Plots cross at same point on y-axis (same V_{max}, different K_t).

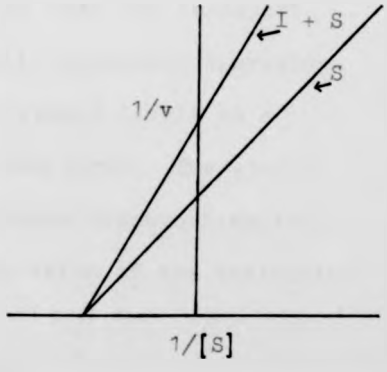


Figure 1.6.4. Non-competitive inhibition. Plots cross at same point on x-axis (same K_t, different V_{max}).

more of the processes outlined above. Methods for establishing the contribution of passive diffusion to the uptake of the test compound are discussed in Section 4.2.3. Mediated transport is normally further distinguished by a demonstration of the inhibition of uptake by structurally-similar compounds. The degree and type of inhibition can be examined by observing the effects of a potential inhibitor, I, on the uptake rate of the test compound. Experimentally, the concentration of I is fixed, and that of S, the test compound, varied, and the results obtained in the presence and absence of inhibitor plotted according to the methods of Lineweaver & Burk. Two outcomes are possible. Figure 1.6.3. represents competitive inhibition, in which test and inhibitor molecules compete for the same membrane binding site(s). This is revealed by a raised K_t value (i.e. reduced affinity) for the test compound but the value of V_{max} does not vary. Figure 1.6.4. shows non-competitive inhibition where a reduced rate of uptake is noted due to the actions of the inhibitor at a level other than the transport binding site, for example on the metabolic machinery operating the transport systems. Such an action reveals itself as a change in the value of V_{max}, but K_t is unaltered. The specificity of the inhibitor for the systems transporting the test compound can be quantified by calculation of the inhibition constant, K_i, from equation (4):-

$$\text{slope of inhibited line} = \frac{K_t}{V_{max}} \left(1 + \frac{[I]}{K_i}\right) \qquad -(4)$$

Where K_t/V_{max} = gradient of line S in Figure 1.6.3., and

I = concentration of inhibitor. A similarity of K_t and K_i values would suggest that both test and inhibitor molecules share the same transport system.

Before proceeding with a consideration of how the kinetic constants K_t, V_{max} and K_i can be used to determine the specificities of transporting mechanisms in the brush border of tapeworms, certain terminology must be clarified. For the purposes of this study it is proposed that the term 'locus' will be used to define a specific point in or on the tegument through which a solute molecule can be transported or adsorbed, and a 'site' defines a specific point in or on a locus at which that molecule may bind. For example, Woodward & Read (1969) demonstrated that the uptake of histidine by adult H.diminuta occurs through two kinetically-distinct loci, but MacInnis & Ridley (1969) showed that the single transport locus for uracil possessed by this tapeworm contains two uracil binding sites.

In general, the surfaces involved in the transport of amino acids possess three separate families of uptake loci, one for neutral, one for basic and one for acidic amino acids, between which there is little interaction, but within which the interactions are extensive (Christensen, 1975; Lerner, 1978; Pappas & Read, 1975). In the Ehrlich ascites cell, the widespread interactions between neutral amino acids at the transport level led to their separation into two groups (Oxender & Christensen, 1963). These two groups were designated 'A' (alanine-preferring) and 'L' (leucine-preferring) and were

considered to represent separate transport loci. Subsequent studies have shown that these cells in fact possess four neutral amino acid transport loci as summarised in Figure 1.7., but the possession of at least A- and L-type loci seems to be a common feature of all transporting surfaces (Christensen, 1975).

The majority of our current knowledge concerning the absorption of amino acids by cestodes has come from studies with the 10 day-old adult H.diminuta, and the following observations are mostly derived from studies with this worm. The absorption of amino acids from the surrounding medium by this parasite was first demonstrated by Daugherty (1957a, b), although the active transport of a solute, in this case glucose, was first demonstrated by Phifer (1960a, b).

The kinetic analysis of amino acid uptake by H.diminuta was undertaken by Read et al. (1963). This study set the standards for investigation into tapeworm nutrient physiology to such an extent that their methods are still employed, and their results frequently quoted, to this day. To briefly summarise their method, male Sprague-Dawley rats of 65-100 g weight were infected per os with 30 cysticercoids obtained from either T.molitor or Tribolium confusum. The worms were flushed from the small intestine 238-244 hours post-infection with 'KRT' saline (Krebs-Ringer saline, buffered to a pH of 7.4 with Tris-maleate; see Appendix 1A for composition). Stunted worms, or worms from infection densities of less than 26 individuals, were discarded. The worms from several rats were pooled, sorted

Figure 1.7. Reactivities of amino acids with neutral amino acid transport systems as defined for the red blood and Ehrlich cell (after Christensen, 1975).

system	Gly	A	ASC	L
major amino acids interacting	gly AIB	pro ala ser thr met	ala ser thr	met leu ile phe
minor amino acids interacting	pro	gly AIB his tyr trp val leu ile phe	pro	gly AIB ala ser thr his tyr trp val
model amino acids		MeAIB		2-ANC

into samples of 5 individuals, and pre-incubated in KRT at 37°C for 30 minutes. At the end of this period, the worms were removed, blotted, and incubated in 4 ml of the desired medium containing the radioactively-labelled substrate at 37°C. Following incubation, the worms were rinsed twice in 50 ml of KRT, blotted and placed in 2 ml of 70% ethanol for the extraction of absorbate. Radioactivity in samples of this extract was determined 24-48 hours later. A major point of controversy concerning this method involves the use of KRT saline. Certain workers, notably Podesta and Mettrick, have drawn the inference that as maleate is an inhibitor of mammalian enzyme action, and Tris competes with sodium ions in certain tissues, KRT saline possibly inhibits the normal functioning of tapeworm membrane transport systems. However, such criticisms have not yet been fully tested experimentally (see Section 3).

Initially, Read and his colleagues examined the absorption of methionine. A summary of their results is given in Table 1.2. The data there presented strongly indicates that the uptake of methionine is by active transport, and, although unaffected by external Na^+ or K^+ or ouabain, is dependent upon pH, impaired by various metabolic inhibitors, and competitively inhibited by all the neutral amino acids tested, as well as histidine, glutamic acid and aspartic acid. The structure of the inhibitor molecule also appears to determine its reactivity with the methionine transport loci, as the absence of the amino group, and its position relative to the carboxyl group, abolish reactivity in the first instance (as shown by the lack of

Table 1.2. Characteristics of the uptake of methionine by 10 day-old adult H.diminuta (after Read et al., 1963)

Time course studies	Non-linear (except for first 5 min). Steady state reached in 40 min.
Mode of uptake	Saturation kinetics. K_t (L-met) = 0.307 mM; K_t (D-met) = 0.368 mM V_{max} (L-met) = 183 µmoles/g/h; V_{max} (D-met) = 111 µmoles/g/h
Accumulation?	Yes. c. 10X medium concentration after 1h
Effects of metabolic inhibitors	INEFFECTIVE: Sodium cholate, eserine, phlorizin, 2, 4-dintrophenol, p-chloromercuribenzoate, estradiol, thyroxine, desoxycorticosterone. EFFECTIVE: Sodium azide, sodium arsenate, iodoacetate.
Effect of pH	Uptake varies over range 4.5-9.0. Maximum uptake at pH = 8.5.
Effects of changing $[Na^+]$, $[K^+]$, ouabain	No effect
Effect of amino acids	Competitive inhibitors of uptake (strongest first): met>thr>leu>ser>ile>ala>val>his>asp >pro >try>tyr>OH-pro>gly>phe>β-ala>glu>lys > arg
Effect of methionine analogues	L-ethionine, DL-methionine sulphoxide, DL-methionine sulfone all inhibited the uptake of L-met.
Effects of compounds similar to amino acids and small peptides	INEFFECTIVE: Homocysteine, taurine, ornithine, citrulline, sarcosine, reduced glutathione, glycylglycine, glycyl-L-leucine, glycylglycylglycine. EFFECTIVE (strongest first): Norvaline, S-methyl-L-cysteine, norleucine, glutamine, 3-amino-L-tyrosine, canavanine, cysteic acid, hippuric acid.
Effect of removing $-NH_2$ group	Methionine hydroxy analogue and N-acetyl-DL-valine had no effect on the uptake of L-met.
Effect of position of $-NH_2$ group relative to carboxyl group	% inhibition of L-met uptake: α-AIB, 89%; β-AIB, 33%; γ-AIB, 0%.

inhibitory action of the hydroxy analogue of methionine, or acetylated methionine or valine), and reduce it with increasing distance in the second (as shown by the decreasing inhibitory action of AIB as the amino group is progressively transferred from the α- to the β- and finally γ- position. Structural analogues of methionine produced significant inhibitions, but peptides were without effect.

The interactions between certain other pairs of amino acids were examined, and from their results, Read et al. concluded that adult H.diminuta possessed four qualitatively different amino acid absorption loci. These were listed as :-

a.) a 'basic' amino acid uptake locus, with which histidine interacts.

b.) a 'dicarboxylic' amino acid uptake locus, with overlapping affinity for monocarboxylic acids.

c.) a 'phenylalanine-tyrosine' locus, with which methionine can interact.

d.) "... at least one other type of methionine locus".

The final locus was an attempt to explain some of the variability in the uptake of amino acids by this worm, and indeed, Read et al. did not dismiss the idea that there might be two or more "other types of methionine loci".

Consequent work has centered on isolating the characteristics of the four loci outlined above. Woodward & Read (1969)

established the presence of a separate 'basic' uptake locus, and found that histidine reacted not only with this locus, but also with a kinetically distinct locus serving for the transport of neutral amino acids. Harris & Read (1968) showed that the non-metabolisable amino acids cycloleucine and α-AIB (Figure 1.9.) entered through at least two loci, one of which was inhibited by proline, and the other by phenylalanine. This suggests that there are at least two loci available for the transport of neutral amino acids. The transport of aromatic amino acids has been investigated by Pappas & Gamble (1980). These authors concluded that at least three loci are responsible for the transport of phenylalanine, tyrosine and tryptophan by adult *H. diminuta*. Two loci are apparently responsible for the majority of phenylalanine uptake, and only one of these is inhibited by glycine, one locus is primarily responsible for tryptophan uptake, and all three loci apparently have equal affinities for tyrosine. In addition, three loci are also utilised by aliphatic neutral amino acids, but not acidic or basic amino acids. Finally, the reciprocal interactions between 15 pairs of amino acids were studied by Mac Innis, Kilejian, Graffe & Read (1976), and revealed the presence of six amino acid transport loci, of which four were concerned with the transport of neutral amino acids (Figure 1.8.). The interactions of tryptophan were not considered in this study, so it is possible that at least one, maybe two, additional loci exist primarily for the transport of aromatic amino acids, as suggested by Pappas & Gamble (1980).

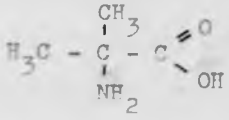
Figure 18

Reactivities of amino acids with amino acid transport systems as defined for H. diminuta (From MaCINNIS et.al., 1976)

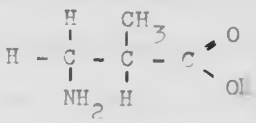
System	Dicarboxylic	Glycine	Serine	Leucine	Phenylalanine	Dibasic
Major amino acids interacting	Asp glu met	gly met	ser ala thr Val pro	leu ile met	phe tyr lis met	Arg lys his
Minor amino acids interacting	ser ala gly	ser thr ala	gly	gly ser thr ala Val	leu ile	

The transport of a molecule by a particular locus appears to be dependent on that molecule possessing certain structural features. It would appear that the free hydrogen atom borne by most amino acids is not essential for transport by the amino acid transport loci as neither cycloleucine or α-AIB possess this feature but both are transported by H.diminuta (Harris & Read, 1968). No evidence is available concerning the importance of the carboxyl group in tapeworm transport locus recognition, but replacement of the carboxyl group by a sulphonic acid group affects neither the rate of transport nor inhibitory activity of the analogue when tested upon the Ehrlich cell (Christensen & Riggs, 1956). However, it is clear that the charge, position and integrity of the amino group are of crucial importance for successful locus recognition and consequent transport in adult H.diminuta. N-methylation of α-AIB (Figure 1.9.) reduces its rate of transport tenfold as compared to the parent compound, although its inhibitory activities are unchanged (Laws & Read, 1969). As mentioned earlier, replacing the amino group with another grouping, or moving it from the α- carbon atom, abolishes recognition in the first instance, and decreases it with increasing distance in the second (Read et al., 1963). At a pH of 7.4, neither oxamic acid nor acetamidoxime (Figure 1.9.) had any inhibitory affect on the uptake of cycloleucine or α-AIB. As these molecules would be uncharged at this pH, the presence of a charged amino group would appear to be important in recognition (Laws & Read, 1969). The length and shape of the carbon backbone of the amino acid molecule is also functionally significant in transport. Oxender & Christensen (1963) showed

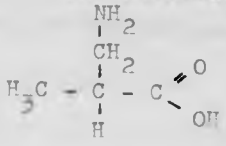
Figure 1.9. Structures of compounds referred to in Introduction



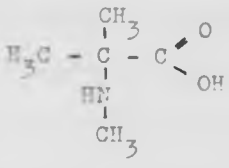
α-Aminoisobutyric acid (α-AIB)



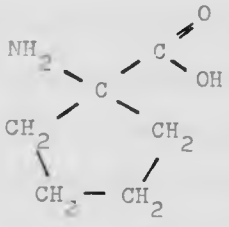
β-AIB



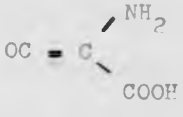
γ-AIB



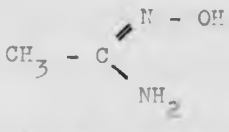
N-methylaminoisobutyric acid (N-MeAIB)



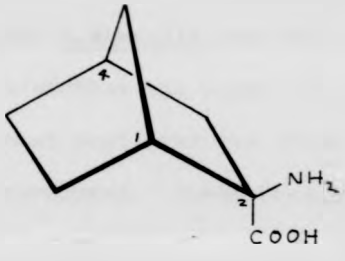
1-Aminocyclopentane-1-carboxylic acid (cycloleucine)



Oxamic acid



Acetamidoxime



(1R, 2R, 4S) form

Absolute configuration

2 - Amino - 2-Norbornancarboxylic acid (2-ANC)
(2 - Aminobicyclo - [2.2.1] heptane - 2 - carboxylic acid)

that a lengthening of the apolar sidechain increased the affinity of the compound for either the 'A' or 'L' loci of the Ehrlich cell. Certain other structural features of molecules also determined affinity for one or other of these loci. For example, a branching of the carbon backbone at either the β- or γ- carbon position decreases the inhibitory action of the molecule on alanine transport (i.e. reduced affinity for the 'L' site). In contrast, the introduction of a polar element on the side chain of an amino acid largely diminishes its ability to inhibit leucine transport. Finally, amino acids with either the α- or β- carbon atoms involved in ring system tend to have dual affinity for both sites (i.e. cycloleucine). The degree to which side chain structure affects locus affinity in tapeworms is considered in Section 5.4.1. Also discussed at that point is the ability of tapeworms to distinguish between stereoisomers of the same amino acid. Other factors which may affect kinetic data obtained with H.diminuta are the age of the worms used, the species of host that the worms are reared in, the infection density within that host, and the time of day at which the experiment was performed. These factors are discussed fully in Section 4.6.

Although the studies described above are valuable in determining the specificity of a transport system, they are of little use in predicting the uptake of one compound from a complex mixture, such as would occur in vivo. Read et al. (1963) derived an equation (5) to predict such an event:-

$$v_i = \frac{V_{max}}{\frac{K_t}{[S]} + 1 + \frac{(K_t)([S_1])}{(K_{t_1})([S])} + \frac{(K_t)([S_2])}{(K_{t_2})([S])} + \ldots \frac{(K_t)([S_n])}{(K_{t_n})([S])}} \quad -(5)$$

Where v_i = velocity of inhibited uptake; V_{max} = maximum rate of uninhibited uptake; $K_{t_{1,2,n}}$ = transport constant of inhibitors; K_t = transport constant of test compound; $[S]$ = concentration of test compound; $[S_{1,2,n}]$ = concentration of inhibitors.

Read *et al.* found that this formula accurately described the effects of specified amino acid mixtures on methionine uptake if K_i, instead of K_t, values for the inhibitors were used. Kinetic data derived from the studies of inhibitory interactions of various amino acids do not necessarily mean that all the amino acids interacting with a particular system are also translocated by that system. It is possible that an amino acid may bind reversibly with a specific transport system but not actually be transported. Such a phenomenon has been termed 'non-productive binding', and is known to occur in the transport of purine and pyrimidine bases by adult *H.diminuta* (Pappas, Uglem & Read, 1973). Read *et al.* (1963) noted differences between the K_t and K_i values when the uptake of one amino acid was studied in the presence of the same (unlabelled) amino acid acting as an inhibitor. Pappas & Read (1975) suggest that the value of K_t is directly related to solute transport, while the K_i value is influenced by both transport and non-productive binding. Thus non-productive binding would be of importance in determining the influx rates of some individual compounds in an amino acid mixture and may explain why K_i values, rather than K_t values, allow the correct prediction concerning the

uptake of a single amino acid from a complex mixture to be made. The equation (5) of Read et al. was also found to accurately predict the absorption of methionine by adult H.citelli from a mixture of amino acids (Senturia, 1964), and the absorption of sodium acetate by adult H.diminuta from a mixture of sodium propionate, butyrate and formate (Arme & Read, 1968).

The relationship of these in vitro experiments to the situation in vivo has been investigated using adult H.diminuta by Hopkins & Callow (1965) and Arme & Read (1969) who studied the efflux of methionine and cycloleucine respectively from pre-loaded worms. Both these studies showed that the efflux rates of either compound were similar whether the pre-loaded worm was surgically reimplanted into the rat intestine or placed in a medium reproducing the in vivo amino acid mixture. Podesta & Mettrick (1974a, b, 1976) have found that the K_t value describing glucose absorption in vivo is much higher than the value obtained by these workers in vitro. Possible reasons put forward by these workers for such discrepancies include the idea that energy availabilty may be rate-limiting in vitro but not in vivo (Podesta & Mettrick, 1974a, b), or the effects of stirring. Wilson & Dietschy (1974) have shown that the presence of an unstirred water layer results in artificially high K_t values for active transport processes. Thus a thick unstirred layer may account for the higher K_t values obtained in vivo. The effects of unstirred water layers, and the effects that they may have upon the data obtained in kinetic experiments will be discussed next.

The methods employed by, and the conclusions drawn by Read and his followers have been criticised by Podesta, Mettrick and their co-workers. The views of the latter workers on KRT have already been remarked upon, but their criticism has so extended as to dismiss most of the results obtained by workers using the methods of Read et al. (1963), primarily due to their failure to take into account the effects of stationary water layers on the rate of solute transport. Thin water films have been found to overlay every biological membrane and their effects upon kinetic measurements have been discussed by Dietschy (1974). To briefly summarise, as a molecule moves, for example, from the intestinal lumen to the cell interior, it encounters two resistances, the unstirred layer, and the membrane itself (Figure 1.10.). In kinetic studies, one sets out to measure the rate limiting step to transport. Normally this is assumed to be the passage of the molecule across the cell membrane, but there may be circumstances where the unstirred layer predominates. For instance, if transport across the cell membrane is very rapid relative to diffusion across the unstirred layer, the passage of the unstirred layer becomes the rate limiting step. Without correcting for the effects of this layer, the value of K_D will be underestimated, and the values of K_t and V_{max} overestimated. The magnitude of this error will depend on the thickness of the unstirred layer, the speed of the membrane translocation process, and the physico-chemical characteristics, primarily water solubility, of the molecule undergoing transport. Podesta (1977a) found that the transport rates of glucose, galactose and alanine across the brush border

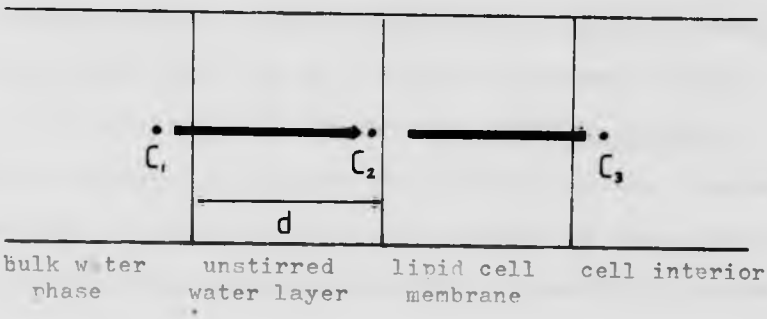


Figure 1.10. Diagrammatic representation of passage of molecule across a biological membrane. C_1, C_2, C_3 = concentration of molecule at three locations. d = thickness of unstirred water layer (after Dietschy, 1974).

membrane of adult H.diminuta were dependent on the thickness of the unstirred layer, and, if not corrected for, the flux rates recorded experimentally would be overestimated, particularly for the more water-insoluble compounds (Podesta, Stallard, Evans, Lussier, Jackson & Mettrick, 1977). By using non-linear transformations to calculate kinetic constants, and correcting for stationary water layer effects, Lussier, Podesta & Mettrick (1982) have resolved a hitherto unknown diffusion component in the uptake of methionine by adult H.diminuta. Although such desires to improve the standards of experimentation are commendable, it appears that the magnitude of these unstirred layers has been considerably overestimated according to recent work involving the rat gut (Smithson, Millar, Jacobs & Gray, 1981) and H.diminuta (Murphy & Lumsden, 1982).

To add to the controversy, there is some doubt concerning the degree of involvement of ions in the transport of amino acids by H.diminuta. Pappas & Read (1975) review the work prior to 1975 and state that ions are not involved, whereas Podesta (1982), primarily on the basis of his own research, argues the contrary position. Podesta's argument is derived from studies in which the inward-facing membrane of the tegument of adult H.diminuta is exposed by slicing the worm lengthways, and the amino acid fluxes through this membrane measured in the presence or absence of ouabain (a cardiac glycoside known to inhibit the transport of Na^+ and K^+ ions, as well as inhibiting the action of the Na^+/K^+-stimulated ATPase of cell membranes) at varying concentrations of Na^+. After correcting for unstirred

layer effects, Lussier (1977) has claimed that loci closely resembling the 'A' and 'L' loci of the Ehrlich cell are present on this membrane, the former having both Na^+-independent and dependent components (the former being both Na^+ and ouabain-insensitive, and the latter being ouabain-sensitive). If one includes the observations of Read et al. (1963) and Podesta, Evans & Stallard (1977) who showed that the influx of amino acids across the brush border membrane is ouabain-insensitive, with the finding that Na^+ transport across this membrane, if not associated with hexose sugar transport, is by passive diffusion alone (Podesta, 1977b), it would appear that the tegument of adult H.diminuta is an excellent example of a functionally-polarised epithelium, with the inward and outward facing membranes both having separate properties. Thus, at the present moment, the findings of Podesta, Mettrick and their co-followers are primarily of interest as manifestations of the membrane physiology of the tapeworm and it is difficult to see how their work can supercede the conclusions summarised by Pappas & Read (1975) concerning the numbers and specificities of the various transport loci possessed by adult H.diminuta.

As previously stated, the aims of this study are to investigate whether the age-dependent morphological changes which occur with cysticercoid development are associated with changes in the pattern of nutrition and whether there are any differences in the basic characteristics of the nutrient transport systems possessed by the cysticercoid and adult worm.

To pursue these objectives, ideally one would select a small group of compounds which interact specifically with one or two transport loci, and then the characteristics of these loci could be compared. Christensen (1975) has found that N-methylaminoisobutyric acid (N-MeAIB) reacts specifically with the 'A' locus, and 2-aminonorbornyl-2-carboxylic acid (2-ANC) (see Figure 1.9.) with the 'L' locus of the Ehrlich cell. However, although H.diminuta appears to possess loci with characteristics similar to the A and L loci of the Ehrlich cell, Laws & Read (1969) found that N-MeAIB did not react solely with an A-type locus. Alternative locus-specific 'model' amino acids were sought by Harris & Read (1968). However, the two synthetic amino acids tested by these workers, α-AIB and cycloleucine, were found to enter the tapeworm through more than one locus, and proline, although previously thought to enter through a single locus (Kilejian, 1966a), was found to interact with a phenylalanine-preferring locus as well as the A-type locus. Thus, at the present time, 'model' amino acids are not available for investigating the characteristics of a specific locus in a particular tapeworm. Section 5.3.6. reports on experiments with N-MeAIB and 2-ANC with the stage V cysticercoid of H.diminuta.

In the absence of suitable 'model' amino acids, it was proposed that the compound to be used in a major part of these studies would be cycloleucine (1-aminocyclopentane-1-carboxylic acid; see Figure 1.9.). This synthetic amino acid has the major advantage of being metabolically inert in many eukaryotic

organisms and appears to enter the cell through neutral amino acid transport loci. The pioneering work with cycloleucine was that of Ahmed & Scholefield (1962), who showed that this compound was absorbed by saturation kinetics and accumulated within slices of rat brain cortex and Ehrlich cells. A consequent study by Oxender & Christensen (1963), also involving the Ehrlich cell, showed that cycloleucine entered the cell through both the 'A' and 'L' loci. This compound is also absorbed through two or more loci by adult H.diminuta (Harris & Read, 1968).

Therefore although not confined to one locus, a study of the uptake of cycloleucine can provide much information concerning the uptake of nutrients by the cysticercoid of H.diminuta. If shown to be metabolically inert in cysticercoids, it can be used as a physiological probe to investigate the changes in the neutral amino acid transport systems of this parasite as it develops. This can be done by monitoring changes in the K_t and V_{max} values describing cycloleucine uptake. Previous studies have shown that cycloleucine can inhibit the uptake of, and be inhibited by, many other amino acids. Thus it is an ideal compound with which to begin an examination of the characteristics of the amino acid transport loci possessed by the cysticercoid.

With the current controversy still surrounding the use of KRT saline for tapeworm in vitro experiments, the first experimental part of this study (Section 3) is concerned with the

testing of a variety of physiological salines and assessing their usefulness for such experiments. Section 4 is concerned with the uptake of cycloleucine by cysticercoids of a variety of ages and developmental stages. Section 5 contains a description of the characteristics of the uptake loci used by cycloleucine and data concerning the uptake of six other neutral amino acids. From the results of this section, a view of the number and specificities of the neutral amino acid transport loci possessed by the cysticercoid can be gained, and this allows direct comparisons to be made with the characteristics of the loci possessed by the adult worm. Section 6 is concerned with protein synthesis by the cysticercoid. In each of Sections 2-6, following an introduction, any theoretical considerations, or methodology not covered in Section 2 (Materials and Methods), will be explained. Each section concludes with a discussion of the results with reference to the relevant literature. Section 7 is an overview of the entire study and concentrates particularly on the role of nutrient transport in the biology of the cysticercoid.

2. MATERIALS AND METHODS

2.1. Maintenance of H.diminuta

2.1.1. Hosts employed

The definitive hosts used in this study were male albino Wistar rats, which were infected *per os* when at a weight of *c.* 100 g with 30 cysticercoids. Post-infection, the rats were housed in standard rodent cages, 2 or 3 to a cage, and allowed to feed *ad libitum* on commercial pelletised rodent food and water.

The intermediate host was the Rice University strain of *Tenebrio molitor* (Coleoptera). The uninfected stock was maintained in bran-filled containers at a constant temperature of 26°C and relative humidity of 80%. Apples and potatoes were occasionally added to the bran as dietary supplements. Infected beetles were fed solely on bran and maintained in an incubator at 26°C. Humidity within the incubator was provided by continually bubbling air into a water-filled trough in the base of the incubator.

2.1.2. Extraction of *H.diminuta* eggs from rat faeces

Host faecal pellets, containing *H.diminuta* eggs, were soaked in water for a minimum period of 24 hours at room

temperature, stirred, and then filtered through a coarse sieve (mesh diameter = 2mm). A large volume of salt was added to saturating concentrations to the filtrate, which was subsequently poured into screw-top plastic bottles and centrifuged once at 1000 g for 10 minutes in a GF-6 Centrifuge (MSE Scientific Instruments, Crawley, England). Following centrifugation, the surface 'scum' of eggs was removed with a syringe and ejected into a large volume of tap water. This water was changed at least twice over a 24-hour period to ensure complete removal of the salt, after which the eggs were removed by filtration, placed in a small volume of distilled water, and stored at 4°C. Eggs so obtained remain viable for at least one month. A more detailed account of egg viability during storage is given by Hundley & Berntzen (1969).

2.1.3. Infection of T.molitor

Adult T.molitor were removed from the stock colony and deprived of both food and water for at least three days prior to infection. Beetles were infected by allowing them to feed on a 1 : 1 mixture of fresh apple pulp and H.diminuta eggs. In some experiments, dried apple powder was used and reconstituted with an exact amount of water containing a known quantity of eggs (see Section 2.4.7.3. for further details). The time that the beetles were allowed to feed had a marked effect on the consequent density of infection. Figure 2.1.3.1. reveals that there is a significant (at $p = 0.05$) difference

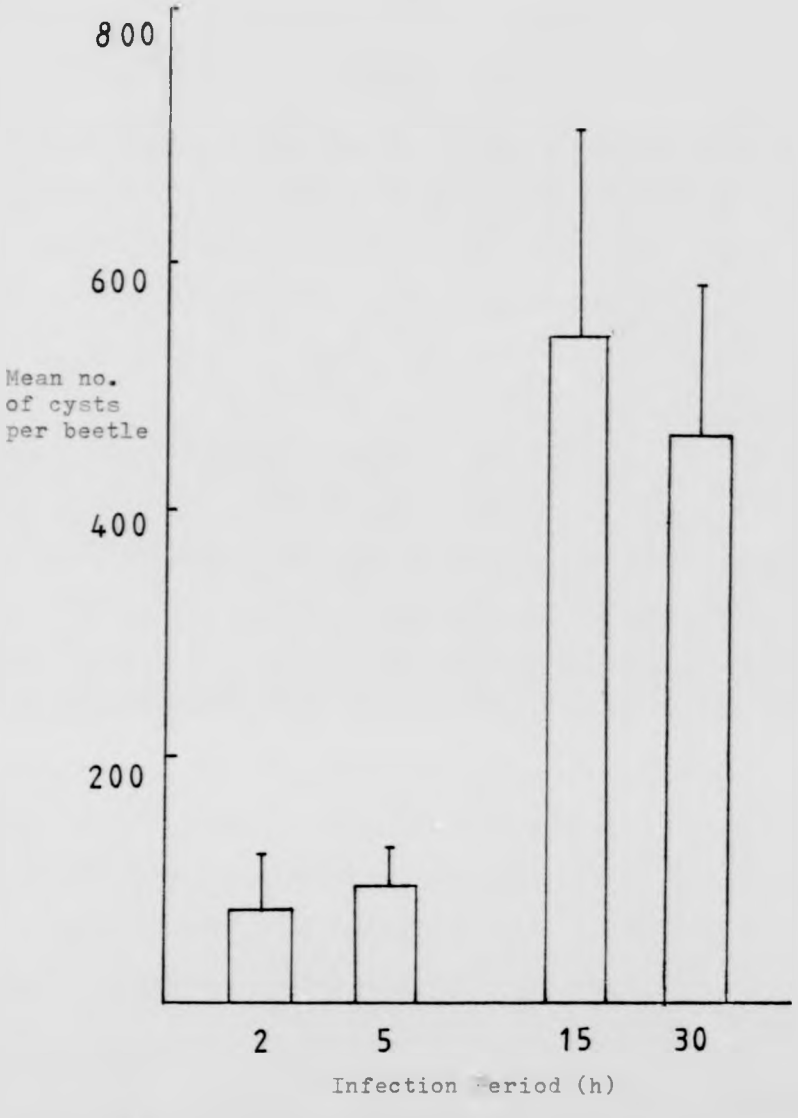


Figure 2.1.3.1. Infection densities of groups of T.molitor left to feed on apple/T.diminuta egg mixes for varying periods of time. Each group of 5 beetles was exposed to $\frac{1}{4}$ of a single batch of apple/egg mix and dissected 16 days post-infection.

between beetles left to feed for 2 or 5 hours and 15 or 30 hours. Routinely, beetles were allowed to feed for 24 hours before the apple/egg mixture was removed.

2.2. Chemicals and Radioisotopes

2.2.1. Chemicals

All the chemicals employed in this study were of Analar or best available grade, with the exception of the salt used in the egg extraction process which was of 'reagent' grade, and were obtained from either BDH Chemicals Ltd., Poole, England, or Sigma London Chemical Co. Ltd., Poole, England.

2.2.2. Radioisotopes

^{14}C-labelled isotopes of a number of compounds were employed in this study. With the exception of ^{14}C-N-methylaminoisobutyric acid (N-MeAIB) which was obtained from New England Nuclear, Boston, U.S.A., all the compounds were obtained from Amersham International PLC, Amersham, England. ^{14}C-L-alanine, ^{14}C-L-leucine, ^{14}C-L-proline and ^{14}C-L-phenylalanine were uniformly labelled with the isotope on all carbon atoms, but ^{14}C-cyclo-leucine, ^{14}C-α-aminoisobutyric acid and ^{14}C-N-MeAIB were labelled at the 1-carbon position only.

Stock solutions were prepared containing the radioisotope and sufficient amounts of the equivalent non-radioactive compound to give a final concentration of 2 mM, and a specific activity of 1 μCi/μmole. This relatively high specific activity was necessary because of the relatively small quantities of

biological material used in each experiment. The stock solutions were kept frozen until use.

Absorbed radioisotope was assessed by the addition of 1 ml of ethanolic extract of radioisotope to 10 ml of the scintillation fluid 'Lumagel' (Lumac Systems Inc., AD Schaesburg, The Netherlands), followed by the counting of this cocktail in a Packard 2425 Tri-Carb liquid scintillation spectrometer on a channels ratio setting. After correction for background radiation, suitable standards were used to allow uptake to be expressed in terms of nmoles radioisotope absorbed/mg dry weight of parasite material/incubation time period.

2.3. Preparation of Cysticercoids for Experimentation

Cysticercoids were removed from the haemocoel of the beetle host as follows; the body of the insect was severed at the thorax - abdomen junction and again at about 2 mm from the posterior end of the abdomen. A saline-filled Pasteur pipette was inserted into the body cavity at the anterior end of the insect and the cysticercoids were flushed into a saline-filled petri dish. After 2 or 3 more flushings, the cysticercoids were concentrated by a gentle swirling of the petri dish. Cysticercoids were rapidly sorted into pools of the desired number, broken, stunted or abnormal cysticercoids being discarded. Care was taken that host debris or other parasites (such as gregarine Protozoa) were excluded. The pools were then removed to the appropriate incubation tube in as small a volume of saline as possible. The time taken to sort 1000 cysticercoids from a larger pool was approximately 15 minutes.

2.4. Development of Experimental Method

2.4.1. Introduction

In previous studies of cysticercoid physiology (Arme & Coates, 1971, 1973) relatively large quantities of parasite material and radioisotope were employed for each replicate experiment. In addition to this, Arme and Coates did not investigate the effects of cysticercoid age, sex of the host, and infection density of the parasite within that host on the uptake of nutrients by the parasite. Consequently, it was felt desirable to develop a technique which was more conservative in its use of materials and would take the possible effects of the variables outlined above into consideration.

Initially, it was felt necessary to select a physiological saline for use in these *in vitro* studies. This subject is fully discussed in Section 3, the conclusions of which suggest that Krebs-Ringer saline, buffered to a pH of 7.4 with Tris-maleate (the 'KRT' of Read *et al.*, 1963) is a suitable medium. All the experiments described in the remainder of this section commence with the pre-incubation of the cysticercoids in KRT for 30 minutes at 26°C. This pre-incubation period has been used in all previous *in vitro* studies concerning *H.diminuta*, and the incubation temperature reproduces the ambient temperature which the cysticercoids will have encountered in the beetle host. The nutrient of choice for the majority of this study is ^{14}C-cycloleucine (see Sections 1 and 2.4.6.) which is presented to the parasites

in KRT saline at 26°C for the desired incubation period.

In developing a suitable technique, the following subjects were investigated (section number in brackets):-

a.) Development of a suitable container to contain the cysticercoids during the experiment (2.4.2.).

b.) A suitable method for washing the cysticercoids post-incubation (2.4.3.).

c.) A suitable method for extracting absorbed radioisotope from the washed cysticercoids post-incubation (2.4.4.).

d.) The number of cysticercoids to be used in each replicate experiment (2.4.5.).

Factors that may affect the uptake of ^{14}C-cycloleucine that could be attributable to the host are explored in Section 2.4.7. The contents of this section are as follows:-

a.) Introduction (2.4.7.1.).

b.) Growth of cysticercoids within Tenebrio (2.4.7.2.).

c.) Presentation of defined numbers of H.diminuta eggs to Tenebrio (2.4.7.3.).

d.) Effects of infection density on growth rate of cysticercoids (2.4.7.4.).

e.) Effects of host sex on growth rate of cysticercoids (2.4.7.5.).

f.) Effects of infection density on the uptake of ^{14}C-cycloleucine (2.4.7.6.).

The section concludes with a discussion (2.5.).

2.4.2. Incubation tubes

A small piece (c. 3-4 cm) of Pyrex glass tubing (bore diameter = 7mm) covered at one end by a mesh of either nylon muslin held in place by a ring of stainless steel wire or 0.2mm mesh diameter stainless steel mesh (obtained from G.W.Heath & Sons (U.K.) Ltd., Burslem, England) stuck in place with an epoxy adhesive was found to be a suitable container for the incubation of cysticercoids. The parasites did not pass through the mesh, and did not rise up the tube and stick to the sides on agitation. The nylon mesh-closed tubes were used for all further experiments in this study, except for those in Section 6 where the steel mesh-closed variant was employed.

2.4.3. Washing procedure

A procedure for washing the incubation tube free of adherent radioisotope following incubation was devised. When empty incubation tubes were subjected to various washing procedures after incubation in ^{14}C-cycloleucine solutions, it was found that a reproducable amount of radioactivity was removed after the tube had been blotted, washed for 10 seconds in 500 ml of KRT and blotted again. The above experiments were repeated with other cycloleucine solutions of concentrations between

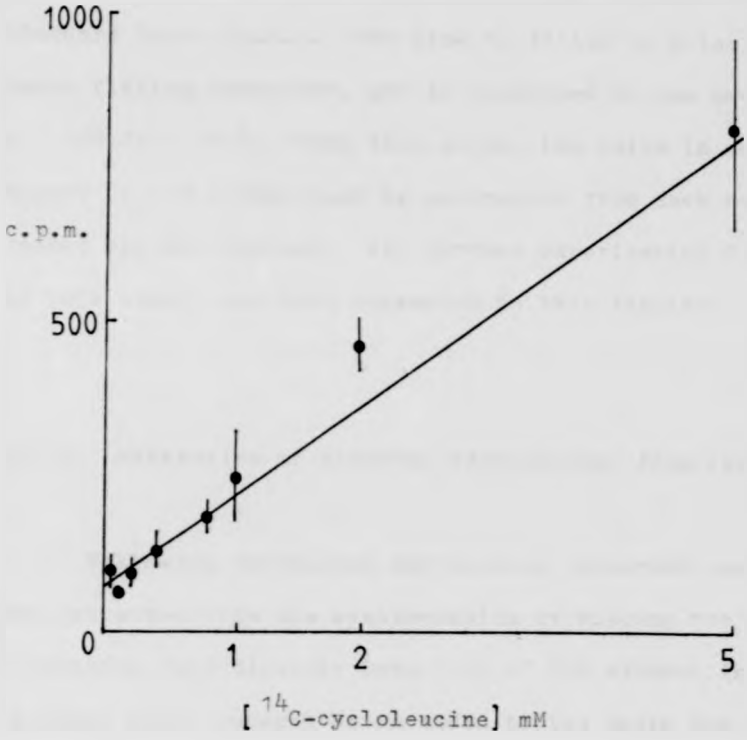


Figure 2.4.3.1. Adherent radioisotope remaining after washing empty incubation tubes incubated in a range of cycloleucine concentrations. Each point is the mean of 10 replicates ± S.E. (for details see text)

0.05 and 5 mM. The results obtained after 30 minutes incubation in ^{14}C-cycloleucine and consequent washing are presented in Figure 2.4.3.1. Each point is the mean of 10 replicates ± Standard Error (S.E.). The line is fitted by a least squares curve fitting procedure, and is described by the equation $y = 166.2x + 70.8$. From this graph, the value in counts per minute (c.p.m.) that must be subtracted from each experimental result can be obtained. All further experimental data obtained in this study have been corrected in this fashion.

2.4.4. Extraction of absorbed radioisotope from cysticercoids

Following incubation and washing, absorbed radioisotope was extracted from the cysticercoids by placing the washed incubation tube directly into 1 ml of 70% ethanol in a scintillation vial, Lumagel being added to the whole the following day.

The above method did not determine the minimum extraction period. A further experiment was thus performed to examine this. Three separate pools of 25 cysticercoids were pre-incubated in KRT, then incubated for 30 minutes in 0.5 mM ^{14}C-cycloleucine, washed, and placed in three separate vials, each containing 1 ml of 70% ethanol. At the end of the first extraction period (30 minutes), the tubes were drained, blotted (the filter paper then being added to this primary ethanolic extract), and extracted for another 30 minutes. At the end of this extraction,

5 further extractions of 1 hour each, and one final extraction of 18 hours, were performed. The amount of radioisotope removed at each extraction is given in Table 2.4.4.1., which clearly shows that the majority of radioisotope was removed in the first 30 minutes, and all of the isotope within 2 hours. A 2 hour extraction period was thus adopted for all further experiments.

2.4.5. Number of cysticercoids to be employed per repilicate

To determine whether the uptake of a nutrient is proportional to the number of parasites, batches of 10, 25, 50 and 100 cysticercoids of varying ages were pre-incubated, then incubated for 30 minutes in 0.5 mM ^{14}C-cycloleucine, washed, extracted in ethanol, and the absorbed radioisotope assayed. The results are presented as Figure 2.4.5.1., which suggests that the uptake of this compound is linear with respect to cysticercoid numbers up to a quantity of 50 parasites. Although an appreciable quantity of cycloleucine is absorbed by 10 cysticercoids, the loss of one or more of them would cause a correspondingly high variation in the final result. A pool of 25 cysticercoids was thus adopted for each replicate experiment.

An additional experiment, performed in an identical manner to that described above, examined the uptake of 0.5 mM ^{14}C-cycloleucine by 10 replicates of 50 cysticercoids that had been previously heated to 60°C for 10 minutes. No detectable

Table 2.4.4.1. Radioisotope recovered from successive ethanol extractions of cysticercoids. Each result is the mean of three replicates ± S.E., corrected for background radiation.

Extraction time	Cumulative extraction time	Radioisotope extracted (c.p.m.)	
		per extraction period	cumulative
30 min	30 min	2433.2 ± 76.6	2433.2
30 min	1 h	91.7 ± 4.8	2524.9
1 h	2 h	15.9 ± 0.7	2540.8
1 h	3 h	0	"
1 h	4 h	0	"
1 h	5 h	0	"
1 h	6 h	0	"
18 h	24 h	0	"

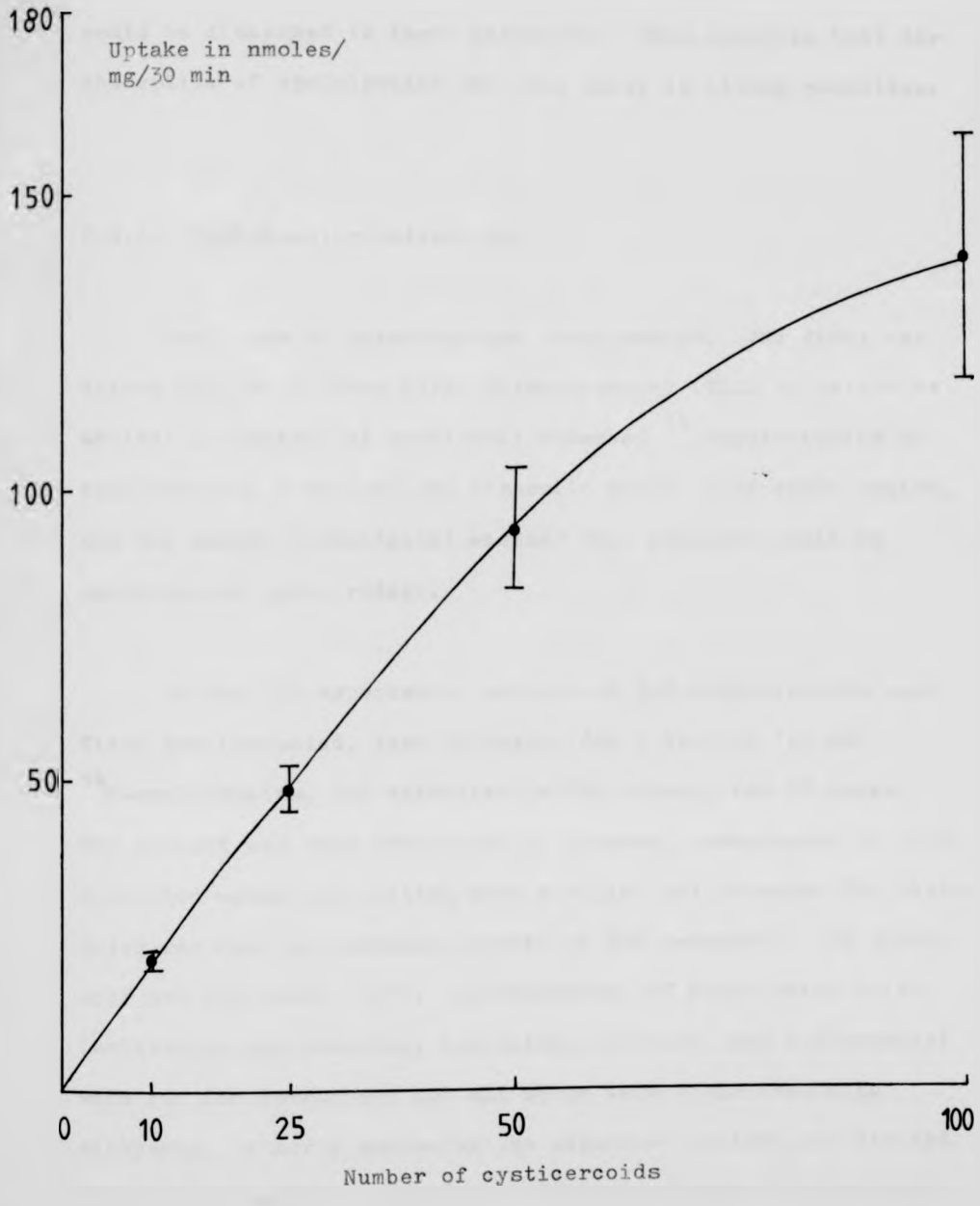


Figure 2.4.5.1. Uptake of ^{14}C-cycloleucine by varying numbers of cysticercoids. Each point is the mean of 10 replicates ± S.E. (see text for details)

radioactivity above that attributable to background radiation could be discerned in these parasites. This suggests that the absorption of cycloleucine can only occur in living parasites.

2.4.6. Cycloleucine metabolism

Two lines of investigation were persued. The first involved the use of Thin Layer Chromatography (TLC) to determine whether an extract of previously-absorbed ^{14}C-cycloleucine by cysticercoids contained any metabolic products of cycloleucine, and the second investigated whether this compound could be incorporated into protein.

In the TLC experiment, batches of 500 cysticercoids were first pre-incubated, then incubated for 1 hour in 1.0 mM ^{14}C-cycloleucine, and extracted in 70% ethanol for 18 hours. The extract was then evaporated to dryness, resuspended in 0.2ml distilled water and spotted onto a silica gel G-coated TLC plate which was run in a solvent mixture of 60% n-butanol, 15% acetic acid and 25% water (v/v). Chromatograms of known amino acids (unlabelled cycloleucine, L-alanine, L-lysine, and L-glutamate) were run for comparison and all spots were visualised with ninhydrin. A strip enclosing the separated extract was divided into 0.5 cm portions, and each of these was assayed individually for radioactivity by transferring the silica gel scrapings into 70% ethanol for 2 hours followed by radioisotope assay using the liquid scintillation technique described previously. In four

replicate experiments, all the radioactivity was recovered from the spot corresponding to unlabelled cycloleucine. Figure 2.4.6.1. is a radioactivity profile of one of these plates.

To test for the incorporation of ^{14}C-cycloleucine into protein, batches of 1000 cysticercoids were pre-incubated, incubated and extracted (to remove unincorporated amino acids) as for the TLC experiment, then homogenised in 5 ml of 5% trichloracetic acid in an ice-cold glass homogeniser for 4 minutes. The homogenate was then decanted into a centrifuge tube, covered with parafilm, and allowed to stand for 1 hour in ice, prior to centrifugation at 2100 g for 15 minutes. The supernatent was discarded and the pellet resuspended in 5% trichloracetic acid and recentrifuged - the entire process being repeated three times. The pellet was then dissolved in 1 ml of distilled water and assayed for radioactivity in the previously-described manner. In three replicate experiments, no detectable radioactivity above that attributable to background radiation could be detected. It was concluded that cycloleucine was not incorporated into protein.

The results of these tests suggest that cycloleucine cannot be metabolised into other amino acids or incorporated into protein. A similar conclusion was also reached regarding the adult H.diminuta by Harris & Read (1968). However, these experiments do not exclude the possibility that cycloleucine is converted into compounds other than amino acids, although the zero count at the chromatogram origin may mitigate against

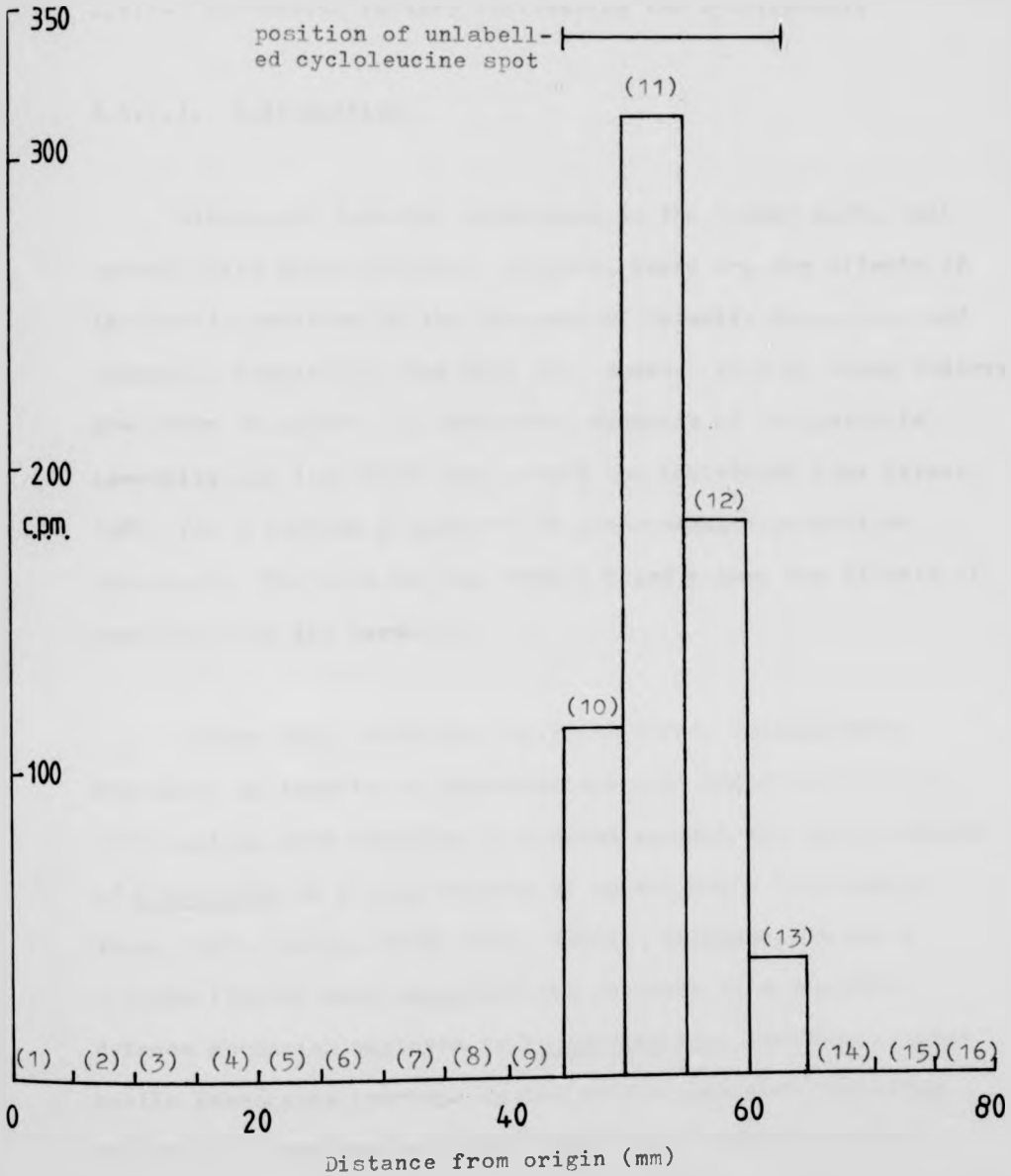


Figure 2.4.6.1. Radioactivity profile of TLC-separated cysticercoid extract (see text for details)

this view.

2.4.7. Biological factors influencing the cysticercoid

2.4.7.1. Introduction

Biological factors, attributed to the insect host, fall broadly into three classes. Firstly, there are the effects of the host's reaction to the presence of invasive parasites, and secondly, competition for food and space. Both of these factors are known to affect the population dynamics of the parasite community and thus must also affect the individual (see Keymer, 1982, for a further discussion of host-parasite population dynamics). To these may be added a third class, the effects of host sex upon the parasite.

Taking host defensive reactions first, encapsulation reactions by insects to parasites are well documented (Nappi, 1975) but no such reaction is mounted against the cysticercoids of H.diminuta in a wide variety of insect hosts (Heyneman & Voge, 1971; Lackie, 1976; Burt, 1980). Ubelaker, Cooper & Allison (1970b) have suggested the presence of a possible defence mechanism employed by H.diminuta cysticercoids against beetle haemocytes (perhaps in the form of branched secretory microvilli), but conclude that there is no effective host resistance to the establishment of the parasite. This has been confirmed by Keymer (1980) who found that when T.confusum was

exposed to successive challenge infections of equal numbers of H.diminuta eggs, the mean parasite burden of the beetle population was directly proportional to the number of parasites introduced.

Several workers have investigated the effects of parasite burden within the host on the growth and development of the cysticercoid of H.diminuta. Most authorities consider that heavy infections lead to a reduction of cysticercoid size (c.f. Schiller, 1959; Dunkley & Wettrick, 1971; Keymer, 1980) although Soltice, Arai & Scheinberg (1971) found no demonstrable effects of crowding. A reduction of size with increased crowding is a noticeable factor in adult H.diminuta populations and the various manifestations of this so-called 'crowding effect' have been well documented (Roberts, 1961, 1966, 1980). A few workers have attempted to quantify these dimension changes. Keymer (1980) found that the total length of H.diminuta cysticercoids maintained in T.confusum fell from 1.5 mm to 0.5 mm as the infection density rose from 1-5 to 71-80 parasites per insect. Voge & Heyneman (1957) consider such size reductions to be solely accounted for by changes in tail length. It is unknown whether such changes occur as a result of competition for nutrient resources or available space. Obviously hosts of a given age, and hence size, will only be able to harbour a finite number of parasites. Before leaving this subject it is worth noting that the site of cysticercoid development within the insect may be dependent upon the density of infection. MacDonald & Wilson (1964) found that low density infections of

H.diminuta cysticercoids (less than 18 individuals) developed in the mesometathorax of T.confusum, whereas higher densities (18-plus) tended to be located in the abdomen.

The literature describing the effects of host sex on parasite growth and population density is somewhat confused. Mankau, Bagwell, Johnson, McLaughlin & Sampson (1971) could find no differences between the susceptibility of male and female T.molitor to H.diminuta but a later publication stated that females are more susceptible (Mankau, 1977). However, it is clear that the cysticercoid of H.diminuta cannot readily develop in either sex of the larval forms of T.molitor and T.confusum (Voge & Graiwer, 1964; Keymer, 1980), unless the hexacanths of this parasite are injected directly into the haemocoel (Voge & Graiwer, 1964). This latter finding would suggest that the barrier to establishment is the gut of larval Tenebrio, and consequent work by Lethbridge (1971a) has shown that the gut wall of larval Tenebrio may be too thick-walled to permit penetration by the oncosphere, but that the gut of adult Tenebrio bears papillae through which the invasive oncosphere may enter the haemocoel (Lethbridge, 1971a, 1980; Ubelaker, 1980).

2.4.7.2. Growth of cysticercoids

Two lines of investigation were persued; weight gains with increasing age, and dimension changes with increasing age.

Considering weight gains first, 100 T.molitor were infected with a single batch of apple/egg mix. At varying times over the next seven weeks at least five beetles were removed and the cysticercoids extracted from them. The parasites were pooled, then counted into a single pool of 1000 individuals, stunted, oversized or malformed examples being discarded. The parasites were dried overnight on pre-weighed squares of steel mesh at 95°C and weighed. This procedure was followed to minimise the effects of infection density on cysticercoid size and hence, total weight, of the sample. The increase of weight with age is plotted as Figure 2.4.7.2.1. The data has been adjusted to give the weight of 25 cysticercoids and is best fitted by a straight line plot described by the equation $y = 0.0014x + 0.0003$ ($r = 0.67$, significant at $p = 0.01$). This graph will be routinely used to express uptake data in terms of μmoles nutrient absorbed/gram ethanol-extracted dry weight/incubation period. It is recognised that such a plot does not show the sigmoidal form normally associated with the growth of adult tapeworms (c.f. growth of adult H.diminuta (Roberts, 1961)). However, the evidence of this section and the ultrastructural studies of Krasnoschekov, Moczoń & Kluzhnikov (1979) and Richards & Arme (1984) suggest that the cysticercoid continues to grow and develop as long as it remains in the intermediate host.

To examine dimension changes, four groups of three beetles were infected from a single batch of apple/egg mix on day 0, each group being exposed to c. 300 eggs. On days 10, 22, 26 and 30, all the beetles in one group were dissected, and 10

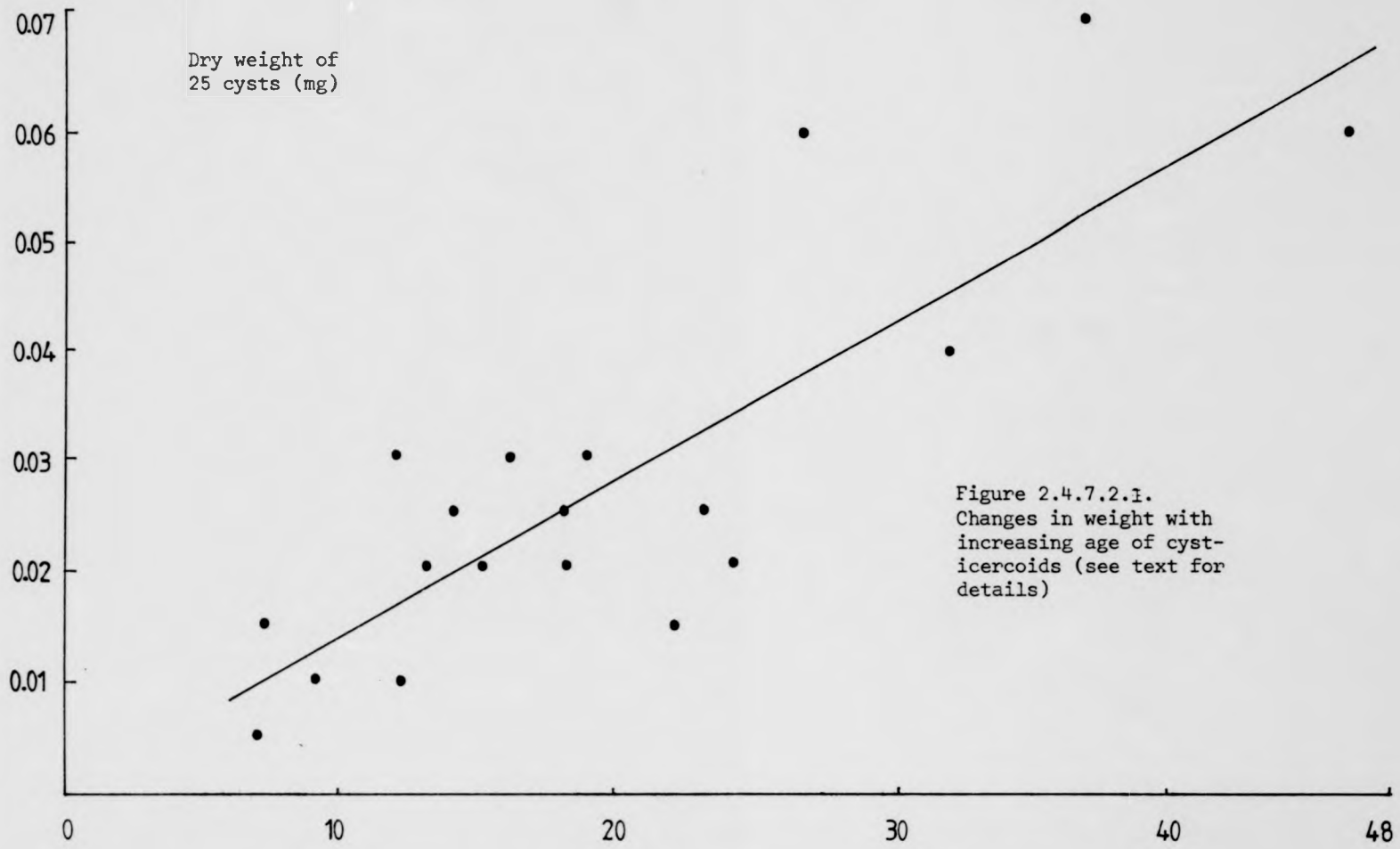


Figure 2.4.7.2.1.
Changes in weight with increasing age of cysticercoids (see text for details)

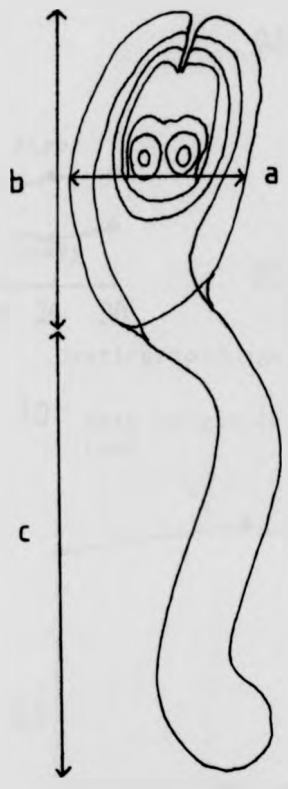


Figure 2.4.7.2.2. Measurements taken on cysticercoids.
a = body width.
b = body length.
c = tail length.

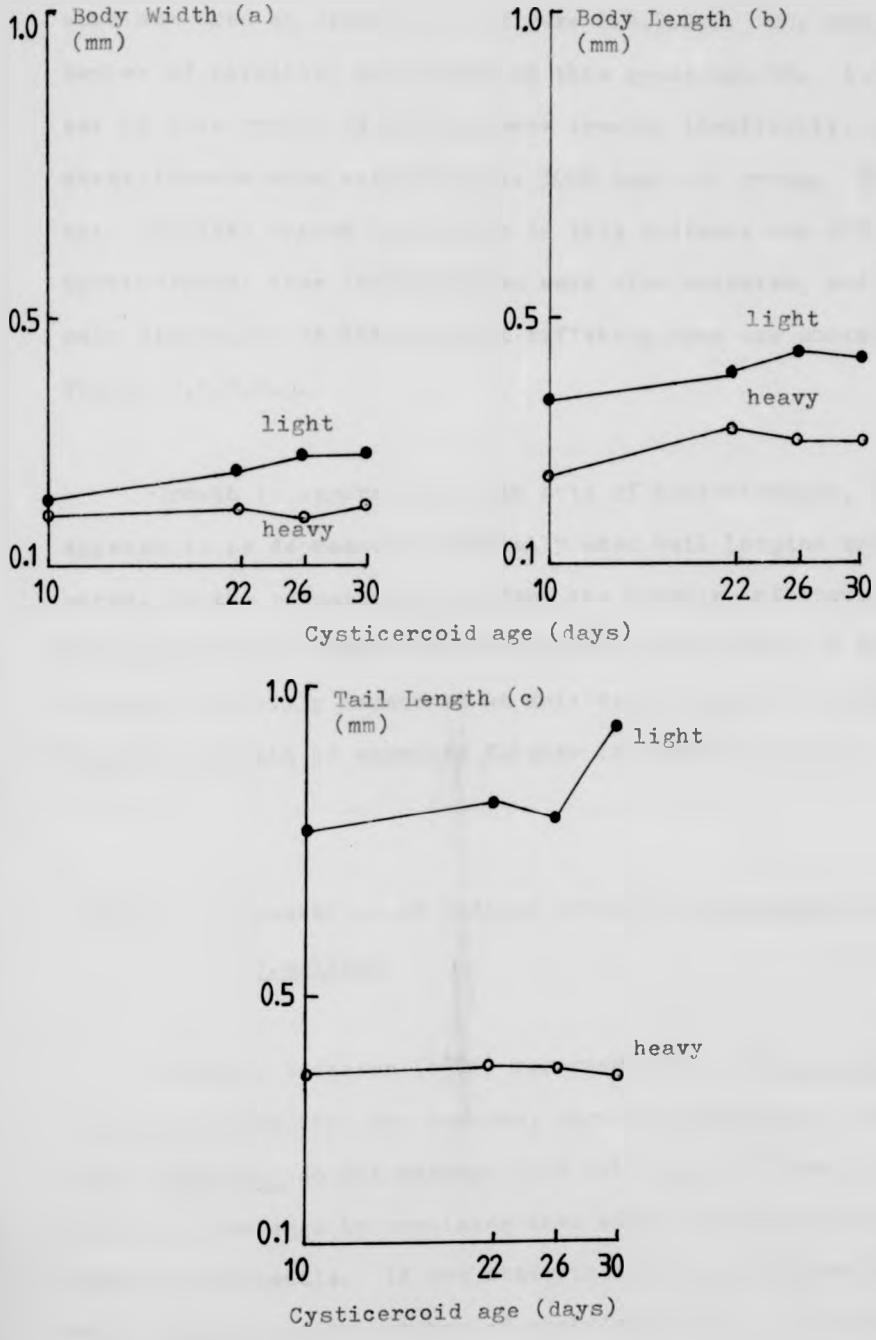


Figure 2.4.7.2.3. Dimensions of cysticercoids of varying ages (see text for details)

individual cysticercoids, selected at random from each beetle, were measured as described in Figure 2.4.7.2.2. The mean number of parasites per beetle in this group was 48. A second set of four groups of beetles were treated identically, only these insects were exposed to c. 3000 eggs per group. The mean parasite burden per beetle in this instance was 605. Cysticercoids from these beetles were also measured, and the mean dimensions of both sets at differing ages are shown in Figure 2.4.7.2.3.

Growth is apparent in both sets of cysticercoids, but appears to be depressed, especially when tail lengths are compared, in the parasites taken from the heavily infected beetles. This section has suggested that growth occurs but that the rate of this is heavily dependent on infection density. Density-dependent growth is examined further in Section 2.4.7.4.

2.4.7.3. Presentation of defined numbers of *H.diminuta* eggs to *T.molitor*

Keymer & Anderson (1979) reported that adult *T.confusum* actively search for, and consume, eggs of *H.diminuta*. However, adult *T.molitor* do not undergo such behaviour and can only be infected with eggs by combining them with a food which is consumed by the beetle. If one attempts to infect beetles individually using separate batches of apple/egg mix, it is found that the liquid from such a mix forms an egg-containing pool around

the mix which is frequently dispersed by the beetles' motions and not consumed. Thus to present defined numbers of eggs to individual beetles, a dried infant food, "Milupa", which contains apple (Milupa Ltd., Uxbridge, England) was employed to bind the egg suspension. When presented to previously starved beetles this mixture was avidly consumed.

A virtually pure preparation of eggs can be obtained by using a modification of the sucrose gradient technique of Lethbridge (1971b). Eggs were separated from rat faeces as described in Section 2.1.2., but instead of adding salt to the coarse filtrate, the filtrate was passed through two sieves, one of 100 µm diameter and the second of 50 µm. The diameter of H.diminuta eggs is c. 70 µm (Lethbridge, 1980), and thus they will be retained by the mesh of the second sieve. The eggs were then scraped from the mesh and resuspended in a small volume of tap water. Three sucrose solutions were prepared and cooled to 4°C. 3 ml of the solution with the highest specific gravity, 2.032 M, was placed in the bottom of a 15 ml centrifuge tube, followed by 3 ml of 1.187 M solution, and finally 3 ml of 1.015 M solution. 4.5 ml of egg suspension was added to the top and the whole centrifuged for 5 minutes at 2100 g. Eggs were removed from the 2.032 M/1.187 M interface, washed in tap water, and then stored at 4°C in a small volume of distilled water. The required concentration of eggs was prepared by dilution, mixed with a little quantity of Milupa, and presented to the beetle.

In two separate tests, 8 male and 8 female T.molitor were individually infected with 300-600 eggs and dissected 22 days later. Of the female sample, 3 were uninfected, but the remaining 5 beetles had a mean parasite burden of 198. Similarly, 2 males were also uninfected, but the other 6 had a mean parasite burden of 209. The "Milupa Technique" was thus employed for the experiments to be described in Section 2.4.7.4.

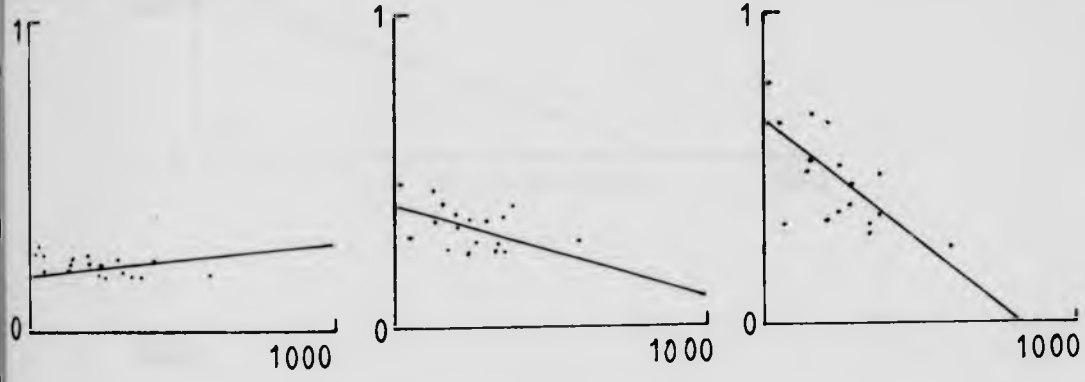
2.4.7.4. Effects of infection density on cysticercoid dimensions

Three groups of beetles were infected individually with c. 300-600 eggs using Milupa. The first group were dissected at 17 days post-infection, the second group after 22 days and the third after 30 days. 10 cysticercoids were selected at random from each beetle and measured as for Figure 2.4.7.2.2. The mean volume of the cysticercoids in each sample of 10 was calculated from the equation $V = (\frac{\pi}{4}) D^2 L$, where D = dimension 'a', and L = 'b'/2. This formula has been used by Soltice et al. (1971) to examine volume changes in cysticercoids. The results are plotted as figures 2.4.7.4.1. (dimension changes) and 2.4.7.4.2. (volume changes). The significance of each plot, as determined by the r values of each analysis, are given in Table 2.4.7.4.1.

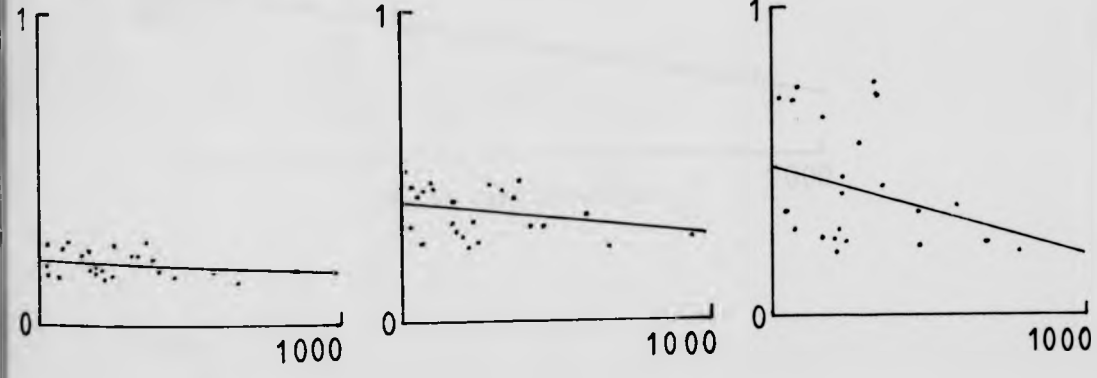
Figure 2.4.7.4.1. Dimensions of cysticercoids at varying infection densities. Each point is the mean of 10 replicates (see text for details). In each graph, the x-axis is the no. of cysticercoids, and the y-axis gives the cysticercoid dimension in mm.

'a': body width 'b': body length 'c': tail length

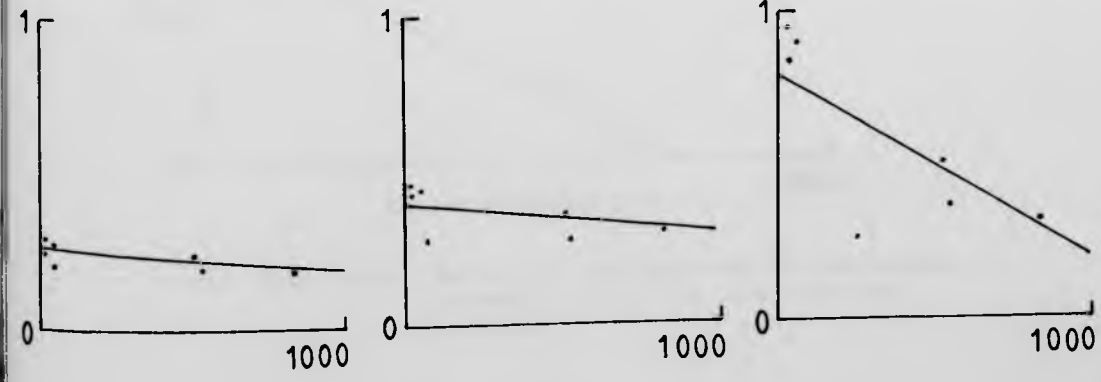
17 day old cysticercoids

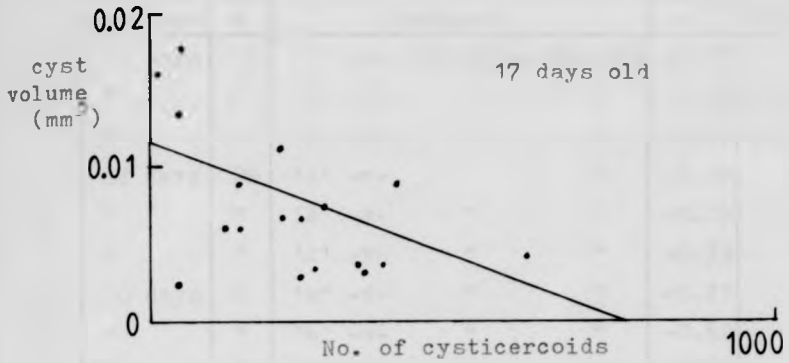


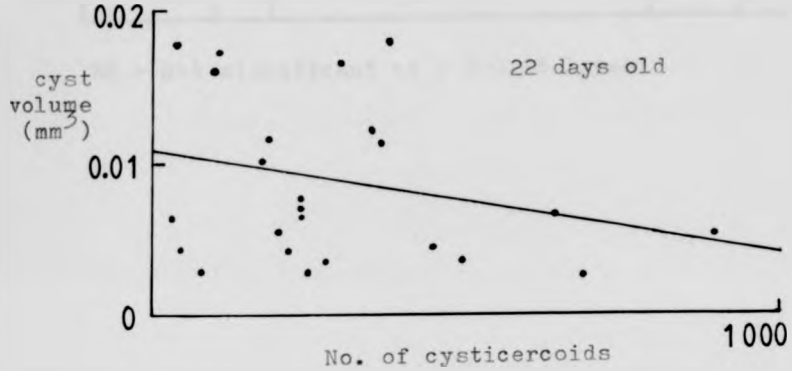
22 day old cysticercoids



30 day old cysticercoids







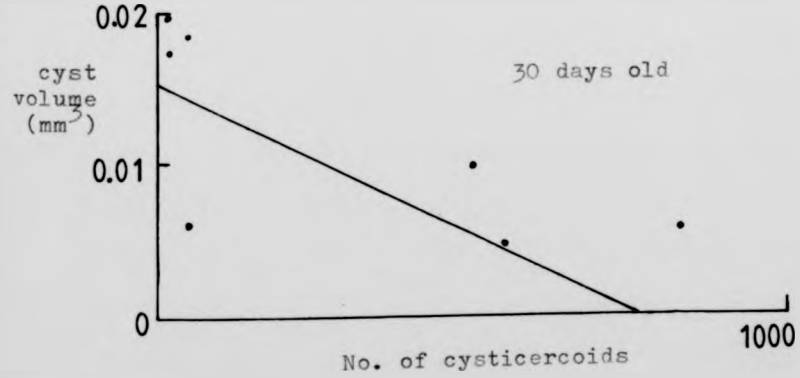


Figure 2.4.7.4.2. Volume of cysticercoids at increasing infection densities (see text for details)

Table 2.4.7.4.1. r values of plots in Figures 2.4.7.4.1. & 2.

Cyst age	n	Analysis	r	Significance
17 days	18	'a' -v- infection density	-0.15	NS
"	"	'b' -v- " "	-0.56	sig. @ p = 0.05
"	"	'c' -v- " "	-0.67	sig. @ p = 0.01
22 days	24	'a' -v- " "	-0.39	NS
"	"	'b' -v- " "	-0.29	NS
"	"	'c' -v- " "	-0.33	NS
30 days	7	'a' -v- " "	-0.71	NS
"	"	'b' -v- " "	-0.62	NS
"	"	'c' -v- " "	-0.67	NS
17 days	18	volume -v- " "	-0.57	sig. @ p = 0.05
22 days	24	" -v- " "	-0.37	NS
30 days	7	" -v- " "	-0.70	NS

NS = not significant at p = 0.05 level

2.4.7.5. Effect of host sex on infection density and cysticercoid development

Table 2.4.7.5.1. presents anew the data of Section 2.4.7.4. concerning 17 and 22-day old cysticercoids on the basis of the sex of the insect host. Insects were sexed, post-dissection, on the basis of their characteristic reproductive organs. The two groups (male and female) are compared by the use of the Student t-test. The weight of the beetles prior to infection was also tested to see if differences between the two sexes existed at this level. The results would indicate that the sex of the beetle host has little effect on the infection density or growth of either 17 or 22-day old cysticercoids. The results also indicate that female beetles are heavier than males.

2.4.7.6. Uptake studies

The uptake of 0.1 mM-1.0 mM ^{14}C-cycloleucine was examined as described in Section 4 and the values of the kinetic constants K_t and V_{max} calculated. 16 beetles were infected with H.diminuta and dissected 11 days post-infection. The cysticercoids from each beetle were sorted into 5 pools of 25 individuals and each pool was incubated for 5 minutes (following pre-incubation) in either 0.1, 0.2, 0.4, 0.8, or 1.0 mM ^{14}C-cycloleucine solution. Values of K_t and V_{max} were calculated for each experiment (16 in total) and the results plotted as scatter diagrams relating them to infection density (Figures 2.4.7.6.1. and 2.4.7.6.2.). The

Table 2.4.7.5.1. Comparisons of beetle weight prior to infection, infection densities and cysticercoid dimensions with respect to the sex of the host

Test	Sex	n	mean ± S.E.	calculated t*	t * p=0.05	signif-cance
weight	♂	25	[illegible] mg	2.048	2.021	sig * p = 0.05
	♀	16	[illegible] mg			
infection density	♂	25	[illegible]	0.011	"	NS
	♀	16	248.1±47.6			
body width(a)	♂	25	0.20±0.01 mm	1.239	"	NS
	♀	16	[illegible]			
body length(b)	♂	25	0.35±0.02 mm	1.617	"	NS
	♀	16	0.31±0.02 mm			
tail length(c)	♂	25	0.48±0.04 mm	1.832	"	NS
	♀	16	0.38±0.04 mm			

* = with 39 degrees of freedom

NS = not significant at p = 0.05 level

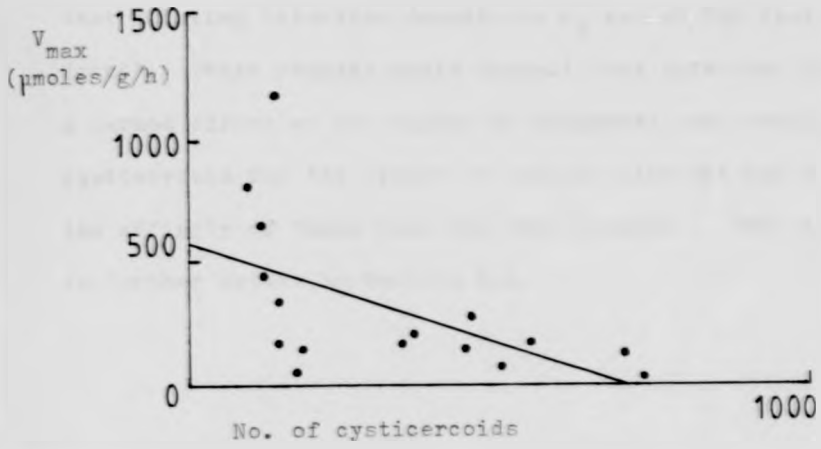


Figure 2.4.7.6.1. Scatter plot relating cysticercoid infection density per beetle to V_{max}

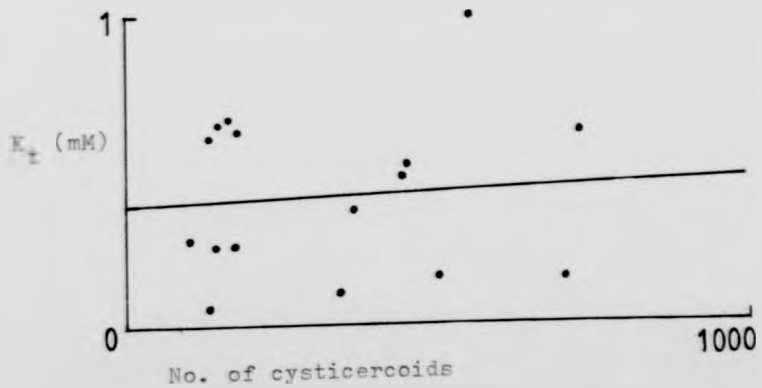


Figure 2.4.7.6.2. Scatter plot relating cysticercoid infection density per beetle to K_t

r value describing the relationship between V_{max} and infection density was calculated as -0.553 (significant at $p = 0.05$), and that relating infection density to K_t was +0.092 (not significant). These results would suggest that infection density has a marked effect on the number of transport loci available to the cysticercoid for the uptake of cycloleucine but has no effect on the affinity of these loci for that compound. This is discussed in further detail in Section 4.6.

2.5. Discussion and Conclusions

This section has developed a method for incubating cysticercoids of H.diminuta in such a way that reproducable uptake data can be obtained. A suitable incubation tube has been designed, and suitable procedures for post-incubation washing evolved. A 2-hour extraction period was deemed sufficient to extract all of the absorbed ^{14}C-cycloleucine from pools of 25 cysticercoids, this amount of parasite material giving a significant result. ^{14}C-cycloleucine is not absorbed by dead parasites, and is not incorporated into protein or metabolised into other amino acids by living metacestodes. The sex of the beetle host Tenebrio had no effect on cysticercoid growth or establishment, but infection density had a growth-retarding effect. This was particularly marked in cysticercoid tail length, and as a consequence of these density-dependant changes, the value of V_{max} fell as the infection density rose. However, the K_t value describing the uptake of ^{14}C-cycloleucine was unaffected by infection density. Possible explanations for the density dependence of V_{max} will be discussed in Section 4.6., but the constancy of K_t augers well for site specificity experiments as it appears to be density-independent.

For the remainder of this study, however, it is proposed that the extremes of infection density, which would produce very large or very small cysticercoids, are to be avoided. This is simply done by using a large enough pool of cysticercoids, drawn from at least five infected beetles, and selecting only those

that appear normally proportioned. At this point in the study, it would appear that V_{max} is not a sufficiently stable parameter to use in comparative studies between different compounds and different organisms. This will be investigated further in Section 4.

3. DETERMINATION OF SUITABLE *IN VITRO* SALINE

3.1. Introduction

As stated in the Introduction (Section 1), the cysticercoid of *H.diminuta* inhabits the haemocoel of a wide variety of insects. In this study, the adult *T.molitor* has been employed as the intermediate host. For *in vitro* studies, parasites must be maintained in a saline that can maintain the normal life processes. Ideally, such a saline should satisfy four criteria:-

a.) It must be ionically and osmotically acceptable to the cysticercoid.

b.) It must contain no substances that would competitively inhibit the uptake of the organic nutrient under study.

c.) It must be stable at the temperature of the experiment, and must not react chemically with the nutrient.

d.) It must maintain the organism in a viable state for an acceptable period of time.

A starting point for the design of such a saline would be a knowledge of the composition of the haemolymph of *T.molitor*. The evidence which is available suggests that the haemolymph of this insect contains a high concentration of amino acids, of which proline is by far the most abundant (Hurd, Browne & Arme, 1982); reducing sugars, of which glucose is the most abundant (Marcuzzi, 1955; Phillips, personal communication); trehalose (Phillips, personal communication); and inorganic ions.

Regarding the last-mentioned constituents, Butz (1957) has measured the concentrations of potassium, sodium and calcium ions, and obtained values of 92.3, 83.5 and 27.1 mg/100 ml respectively. A similar study by Hurd (personal communication) found the concentrations of these ions to be Na^+, 155 ; K^+, 36.4 ; Ca^{2+}, 21.2 ; Mg^{2+}, 22.4 (all values in mmoles/mg. Butz also gives the molarity of adult Tenebrio haemolymph as isotonic to 0.2 molal NaCl, and 0.14 molal $CaCl_2$. Hurd (personal communication) has determined the osmotic pressure of the haemolymph to be c. 500 mOsmol. Butz (1957), in a study of the physiology of the isolated heart of T.molitor, found that this organ was tolerant of a wide range of ionic conditions. When the Na^+/K^+ ratio of the bathing medium was the same as that of the adult (0.9), the heart beat was regular, and lasted for 24 hours. The Na^+/K^+ ratio could be varied from 0.9-14.4 without any appreciable effects. A similar lack of effect was noted when the K^+/Ca^{2+} ratio was varied from 0.5-3, but when potassium was increased to 4, the heart beat became irregular, and the organ did not continue beating for 24 hours. A concurrent study found that the normal rate of heart beat was best maintained over the 24-hour test period in a solution of between $\frac{3}{4}$ and $1\frac{1}{4}$ times normal osmotic pressure.

This relatively high Na^+/K^+ ratio is similar to the ratio found in vertebrate sera, although other groups of insects (viz: Hymenoptera, phytophagous Lepidoptera and phytophagous Coleoptera) have lower Na^+/K^+ ratios (Buck, 1953). These latter findings are reflected in the compositions of the various salines, mammalian

salines such as Hanks' having high Na^+/K^+ ratios, and insect salines such as Hoyle's, Grace's or Schneider's a lower ratio.

Most workers concerned with the membrane transport of nutrients by adult H.diminuta have used Krebs-Ringer saline, buffered to a pH of 7.4 with 25 mM Tris-maleate (the 'KRT' of Read et al., 1963). However, certain workers have opposed the use of this saline for such studies on the grounds that maleate is an inhibitor of mammalian enzyme action, and Tris is a competitor for Na^+ receptors. It is a matter of some dispute whether these criticisms are valid, as to extrapolate the effects of maleate upon mammalian enzymes to helminth transport mechanisms may be erroneous, and although Na^+ ions are involved in the absorption of carbohydrates by cestodes they have not yet been conclusively proved to be involved in the transport of amino acids across the brush border membrane of these parasites (see Introduction, Section 1, for further discussion).

In this study, the suitability of four salines, KRT, Hanks', Hoyle's and KRP (phosphate-buffered Krebs-Ringer saline) salines were investigated. When examining the extent of their effects (if any) upon the cysticercoid, such criteria as egg-laying or movement, as have been used to assess the viability of schistosomes in vitro (discussed by Shaw & Erasmus, 1974) obviously cannot be used. For the purposes of this study, five lines of investigation were followed:-

a.) Infectivity *in vivo*. Cysticercoids were incubated in each saline for a fixed time, fed to adult rats, and the percentage developing to adult worms determined.

b.) Excystation *in vitro*. Cysticercoids were incubated in each saline for a fixed time, excysted with the appropriate enzymes, and the percentage successful excystations with each saline noted.

c.) The ultrastructure of cysticercoids following incubation in the four salines was compared to that of control cysticercoids fixed immediately after recovery from the beetle host.

d.) Uptake studies. The pattern of uptake of ^{14}C-cycloleucine made up in each of the four salines, following pre-incubation in the same saline, over a 30 minute period was compared.

e.) Protein synthesis studies. Following a 1 hour incubation in a medium containing ^{14}C-L-leucine made up in the appropriate saline, the amount of protein synthesised during this period was determined and compared.

3.2. Materials and methods

3.2.1. General considerations

The compositions of the four salines are given in Appendix 1A. Hanks' and Hoyle's salines were made up at 1X strength. Krebs-Ringer (KR) saline, 0.25 M Tris-maleate buffer (TM) and 0.1 M phosphate buffer were made up at 10X strength. All solutions were stored at 4°C until required. KRT and KRP salines were made up freshly by mixing 1 part KR with 1 part buffer and 8 parts of water. Cysticercoids were obtained as described in Section 2.3. In the case of experiment 3.2.3., the salines were pre-warmed to 37°C.

3.2.2. Infectivity *in vivo*

Young male Wistar rats of c. 100 g each were infected *per os* with a known number of cysticercoids that had been previously incubated in one of the four salines for a defined period of time. Twelve days post-infection, the worms were removed from the rat and the percentage establishment per rat determined.

3.2.3. Excystation *in vitro*

The method of Rothman (1959) was employed. Cysticercoids were flushed from the body cavity of the beetle host 11-15 days

post-infection using the appropriate saline. 5 replicates of 50 individuals were pre-incubated in this saline for 1 hour at 26°C, then excysted by treatment with pepsin/HCl (0.1 g pepsin (activity 1 : 2500), 0.5 ml 10 M HCl made up to 100 ml with saline) for 30 minutes, followed by 30 minutes in bile/trypsin (0.25 g sodium tauroglycocholate, 0.1 g trypsin (activity 1 : 10000) made up to 50 ml with saline). The number of successful excystations over the next hour was recorded. A further experiment determined the number of successful excystations from a single batch of 100 cysticercoids pre-incubated for 24 hours in the appropriate saline. Both enzyme solutions were made up in the saline under investigation, and all experiments were carried out at 37°C.

3.2.4. Electron microscopy

Cysticercoids aged between 13-18 days were flushed from the haemocoel of the beetle host using one of the four salines. After varying incubation times in the same saline at 26°C, the parasites were processed for electron microscopy using the following procedure (control cysticercoids, aged 18 days, were flushed from the haemocoel of the beetle host with 4% Millonig-buffered gluteraldehyde and fixed directly):- The parasites were fixed in 4% Millonig-buffered gluteraldehyde overnight, then washed 3 times with Millonig's washing buffer, post-fixed in 1% Millonig-buffered osmium tetroxide for 2 hours, washed twice in distilled water, dehydrated in an acetone gradient

(10 min in 30%, 10 min in 60%, 10 min in 90% and 2X 10 min in 100%) and finally, embedded in Spurr's Resin. Ultrathin sections were cut using an LKB ultramicrotome, stained with uranyl acetate and lead citrate and examined using a Philips 200 electron microscope. The composition of the solutions used for these experiments are given in Appendix 1D.

3.2.5. Uptake studies

The uptake of 0.5 mM ^{14}C-cycloleucine (specific activity = 1 μCi/μmole) was determined over a 30 minute period. Batches of 25 cysticercoids, aged 26-28 days, were pre-incubated in one of the four salines for 30 minutes at 26°C, then incubated for 1, 2, 5 or 30 minutes in a cycloleucine/saline solution at 26°C. 5 replicate batches of cysticercoids were employed per time period per saline. At the end of the incubation period, the cysticercoids were washed and the absorbed radioactivity assayed as described in Section 2.4. By the use of suitable standards, uptake was expressed in terms of nmoles ^{14}C-cycloleucine absorbed/mg ethanol extracted cysticercoid dry weight/incubation period.

3.2.6. Protein synthesis studies

Batches of 1000 cysticercoids were incubated for 30 minutes, then incubated for 1 hour in 0.1 mM ^{14}C-L-leucine made up in the appropriate saline. The post-incubation treatment of the cysticercoids to determine the amount of leucine incorporated into

protein is described in Section 6.2. The total amount of ^{14}C-L-leucine-containing protein formed in 1 hour was expressed as nmoles L-leucine incorporated into protein/mg ethanol extracted cysticercoid dry weight/hour, and the unincorporated (free pool) leucine as nmoles/mg/hour.

3.3. Results

3.3.1. *In vivo* experiments

The percentage establishment rates of the experimentally-infected rats are presented in Figure 3.3.1.1. Separate experiments, not connected with this study, have shown the percentage establishment of *H.diminuta* cysticercoids, not kept in saline for anything but the period of time to sort them into pools of 30 individuals, to be 87-100%. Examination of Figure 3.3.1.1. reveals little difference in the infectivity of cysticercoids kept for 1, 3 or 24 hours in KRT or KRP saline. Only a few adult worms were recovered from rats if the cysticercoids had been pre-incubated in KRP saline for 72 hours, and none were recovered if the cysticercoids had been pre-incubated in KRT saline for the same length of time. Incubation of the cysticercoids in KRT or KRP saline for 120 hours rendered them non-infective to rats. There was no statistically significant difference between the infectivity of cysticercoids that had been maintained for 24 hours in either KRT, KRP or Hanks' salines, but cysticercoids that had been maintained in Hoyle's saline for 24 hours showed a significantly higher (@ $p = 0.05$) infectivity.

3.3.2. *In vitro* excystation

The percentage successful excystations after 1 or 24 hours

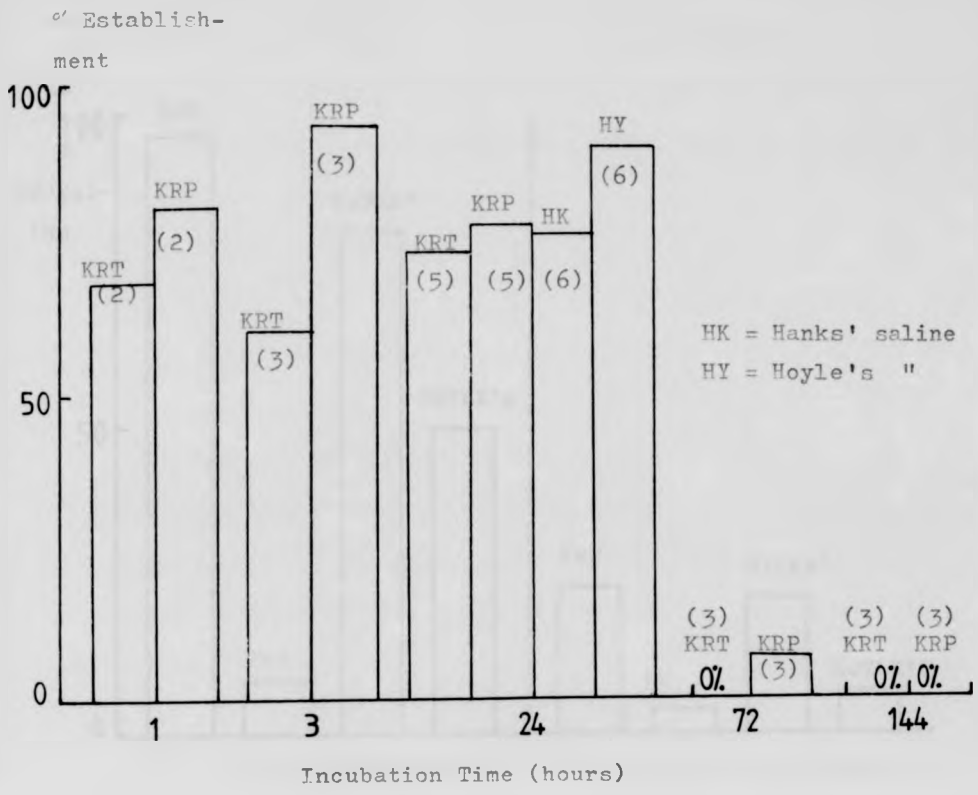


Figure 3.3.1.1. Mean percentage infection densities of rats infected with H.diminuta cysticercoids which have been previously incubated in various salines for varying periods of time. Figure in brackets in each histogram block is the number of replicates per experiment.

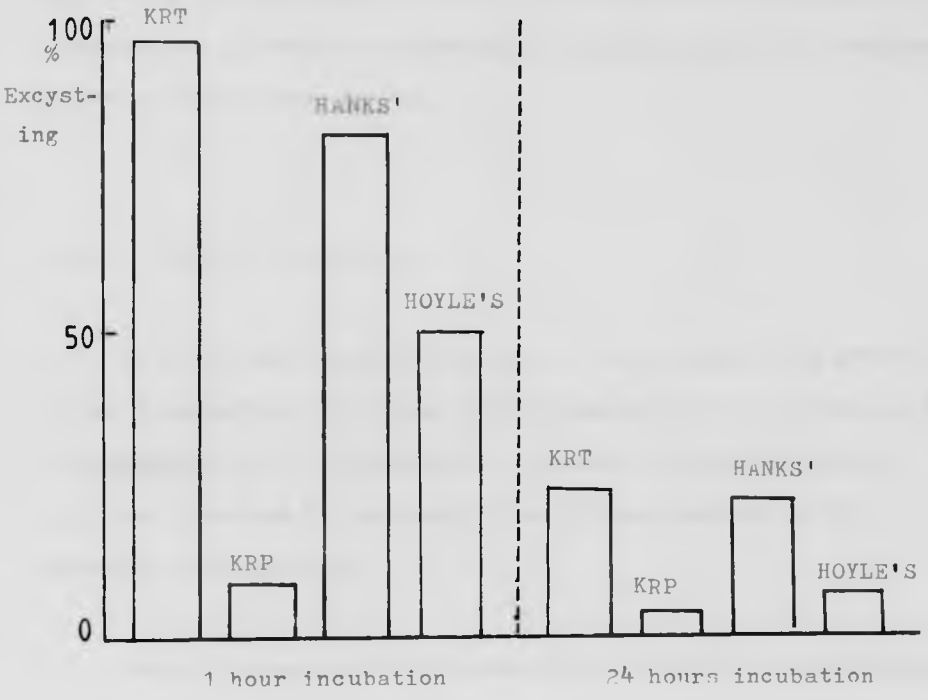


Figure 3.3.2.1. Mean % successful excystations of cysticercoids which have been pre-incubated in one of four salines prior to treatment with excysting enzymes at 37°C. Each histogram block represents the mean of 5 replicate experiments (1 hour pre-incubation) or 1 experiment (24 hours pre-incubation).

incubation in all four salines is presented in Figure 3.3.2.1. The saline that supported the highest rate of successful excystations was KRT, followed by Hanks', Hoyle's and finally KRP. This trend was repeated in the 24 hour incubation experiments, although the percentage excystation in this instance is considerably less than after 1 hour.

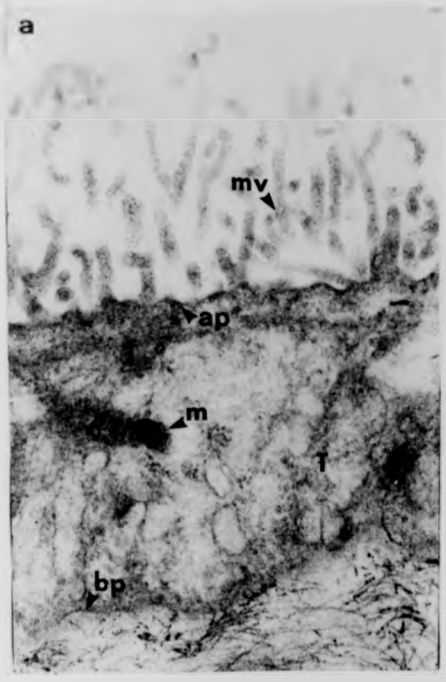
3.3.3. Electron microscopy.

In order that comparisons can be made between the effects of the four salines upon the ultrastructure of the cysticercoid of H.diminuta it is necessary to consider in greater detail than that provided by Section 1. the ultrastructure of the untreated cysticercoid.

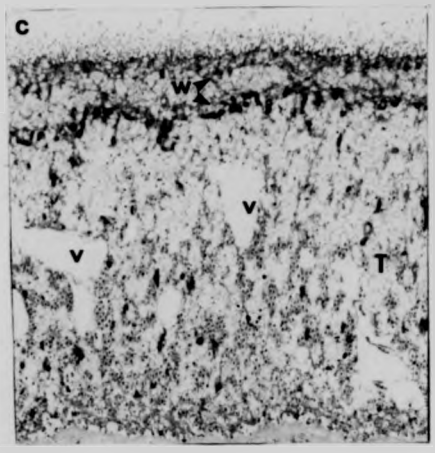
Voge & Heyneman (1957) used five stages (1-5) to describe the morphological appearance of the cysticercoid from the early spherical form (Stage 1) to scolex retraction (Stage 5). Even within this series the authors found it necessary to refer to 'early', 'middle' and 'late' stages (for example, their stages 2 and 3). However, after scolex retraction, the 'capsule' (original mid-body of the 3rd stage) enlarges and 'hairy processes' (Voge, 1960) become apparent. Ultrastructural studies of the cysticercoid wall, after scolex retraction, have been performed on specimens of various ages from differing hosts maintained at different temperatures (Ubelaker, Cooper & Allison, 1970a; Allison, Ubelaker & Cooper, 1972; Cooper, Allison &

Ubelaker, 1975; Krasnoschekov, Moczoń & Pluzhnikov, 1979; Ubelaker, 1980). Comparisons between these studies are therefore difficult, and as a result of this, Richards & Arme (1984) have carried out an extensive ultrastructural analysis of the development of the cysticercoid body wall of H.diminuta derived from Tenebrio that have been maintained at 26°C from 2-46 days post-infection. These workers stressed that the major morphogenetic changes occur after scolex retraction, and therefore found it neccessary to devise an alternative series of developmental stages, designated I-XI, with stage VI denoting scolex retraction.

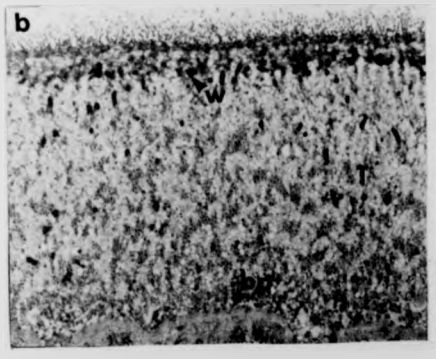
Of the four tissues found within the cysticercoid body wall (tegument, muscle, fibroblasts and associated fibrils and inner cysticercoid tissue) the tegumentary and fibrous systems showed marked changes after scolex retraction. The former is therefore pertinent to the present study. Before retraction, the syncytial superficial cytoplasm of the tegumentary system of the mid body (which becomes the capsule region of the retracted cysticercoid) remains shallow (c. 0.4 µm), but after retraction there is a rapid increase in depth and complexity (various 'webs' appear, and there is an increase in the numbers of mitochondria present in this region), followed in stages X and XI by senescence and pronounced vacuolation. Concurrent with this, is the extracytoplasmic 'intrusion' of fibril bundles from the fibrous system into the tegument syncytial superficial cytoplasm, carrying with them basal plasma membrane infoldings. These fibril bundles become very prominent after about 25 days



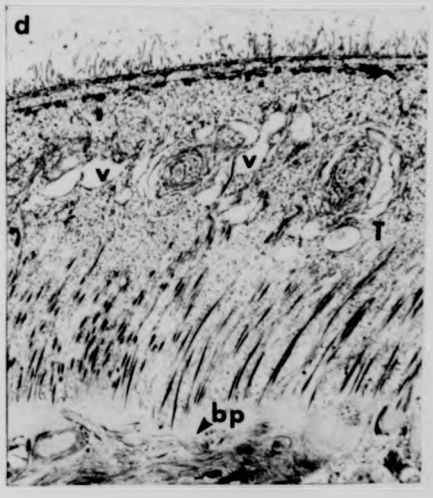
a.) 8-day old cysticercoid



c). 18-day old cysticercoid

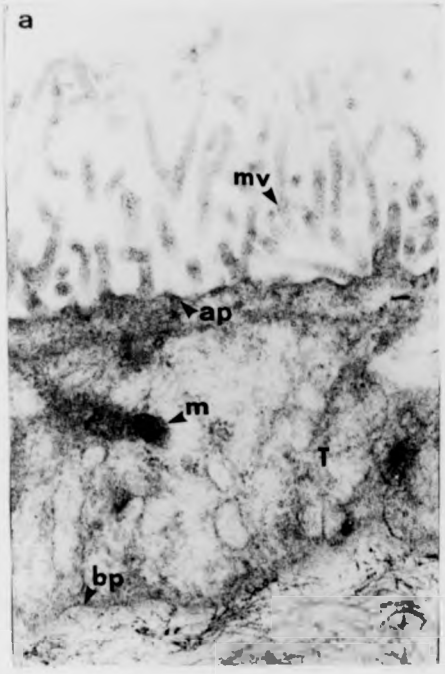


b.) 13-day old cysticercoid

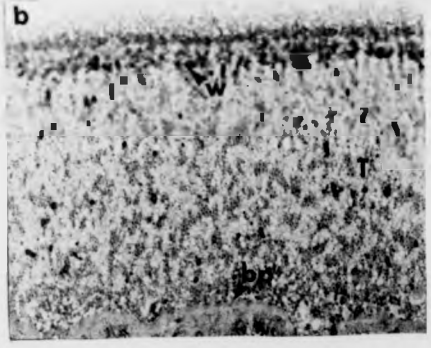


d).31-day old cysticercoid

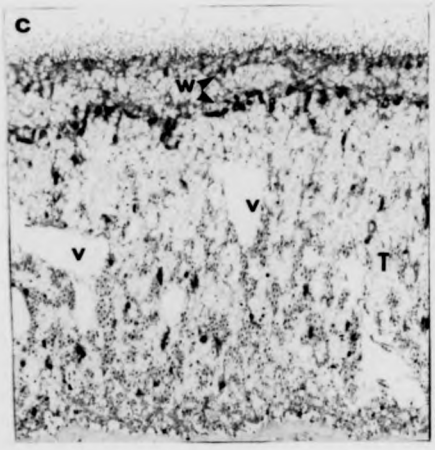
Plate 3.3.3.1. Changes in the tegument of the mature (infective) cysticercoid as it ages. Prints b, c, and d X3570, print a X42,100. See text for further details.



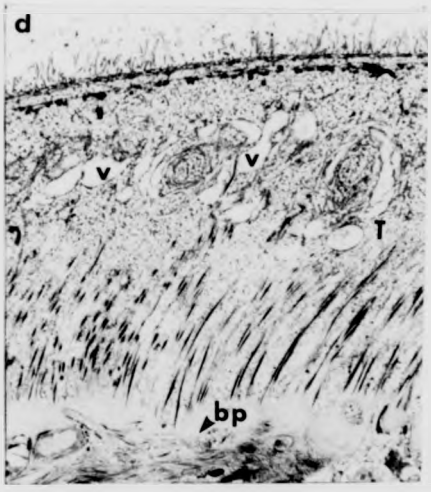
a.) 8-day old cysticercoid



b.) 13-day old cysticercoid



c). 18-day old cysticercoid



d).31-day old cysticercoid

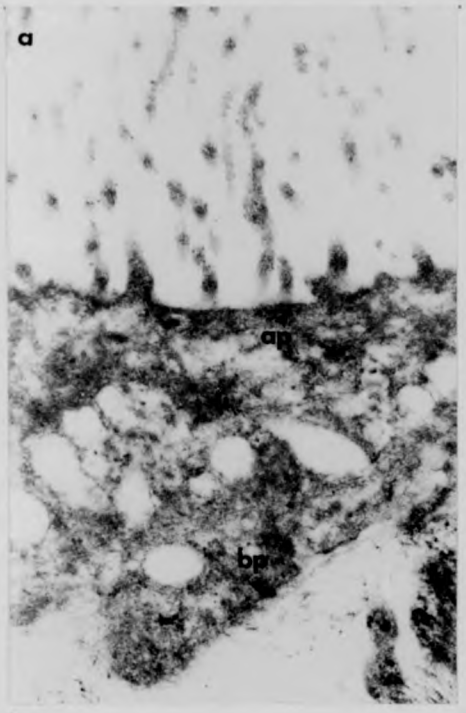
Plate 3.3.3.1. Changes in the tegument of the mature (infective) cysticercoid as it ages. Prints b, c, and d X3570, print a X42,100. See text for further details.

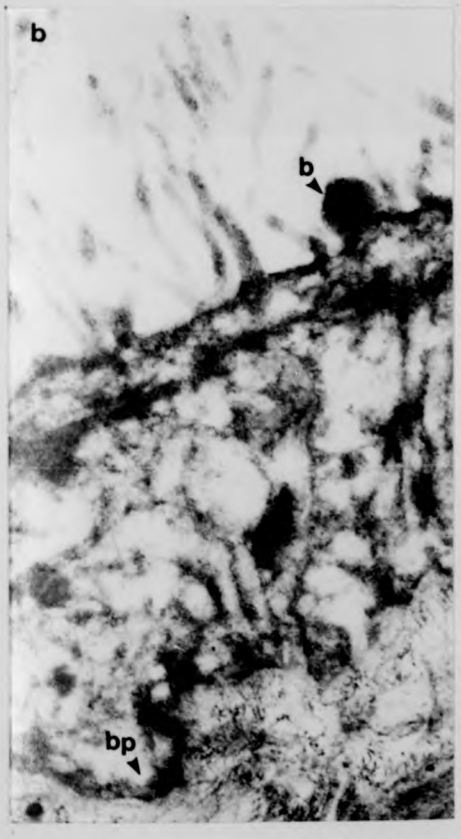
post-infection, and are considered to represent the 'hairy processes' described by Voge (1960). However, microvilli are present throughout the ageing of the cysticercoid and the apical plasma membrane does not show changes.

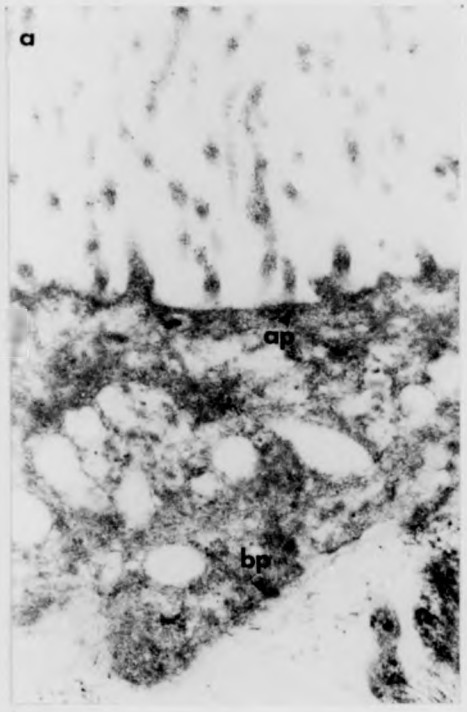
Plate 3.3.3.1. shows the changes in the tegument of the capsule of the mature (infective) H.diminuta cysticercoid as it ages. Print (a) shows the 8 day-old cysticercoid, (b) the 13 day-old, (c) the 18 day-old and (d) the 31 day-old. It is immediately apparent from these micrographs that the microvilli (mv) are retained throughout life, and measurements taken from micrographs of higher magnification show that the width of individual microvilli vary between 0.05 and 0.07 µm. Although the resolution of these micrographs is poor, a study of the specimens depicted in Plate 3.3.3.1., and the higher magnification micrographs referred to above, reveals that the integrity of the apical (ap) and basal plasmalemma (bp) is maintained throughout the life of the cysticercoid. As the cysticercoid ages, the tegument (T) increases in depth, while webs (w) and mitochondria (m) become apparent. The cytoplasm of the tegument also becomes vacuolated with increasing age. Vacuoles (v) are apparent in the 13 day-old specimen, but are particularly marked in the older specimens (prints (c) and (d)).

Plate 3.3.3.2. shows the appearance of the tegument of cysticercoids that have been incubated in KRT or KRP salines for 1 hour. Print (a) shows an 8 day-old cysticercoid that has been incubated in KRT saline, (b) a specimen of the same age

Plate 3.3.3.2. Changes in the tegument of the 8-day old cysticercoid (prints (a) and (b)) or the 30-day old cysticercoid (prints (c) and (d)) after 1 hours incubation in either KRT (prints (a) and (c)) or KRP (prints (b) and (d)) saline. Prints (a), (b) and (d) X42,100, print (c) X72,300. See text for further details.







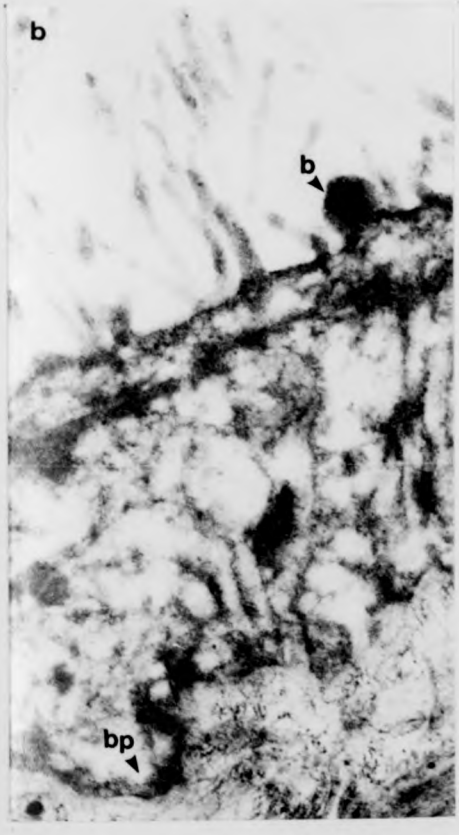
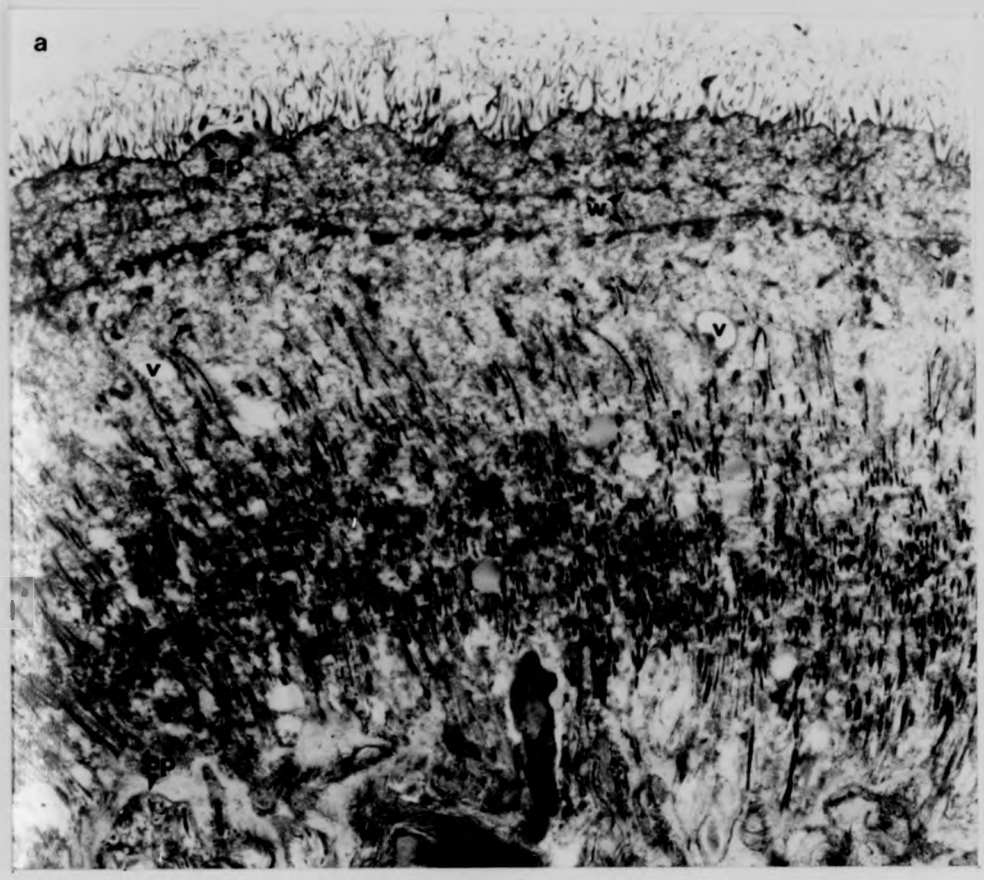


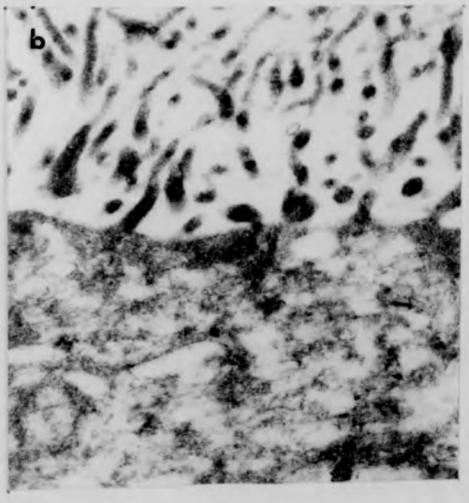
Plate 3.3.3.3. Changes in the tegument of the cysticercoid after 3 hours incubation in RPT, Hanks' or Hoyle's salines.

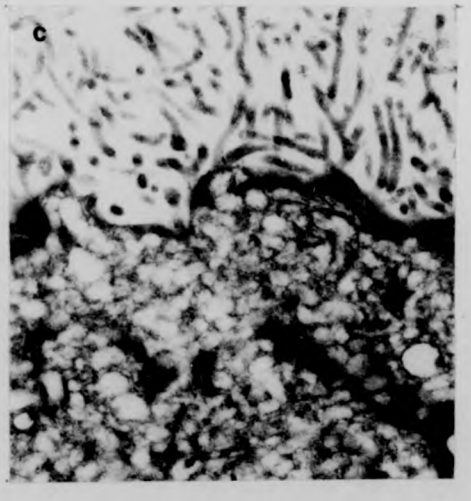
Print (a). 3 hours in RPT (X4463). This micrograph shows the entire depth of the tegument of this 30-day old cysticercoid, and should be compared with print (d), Plate 3.3.3.4.

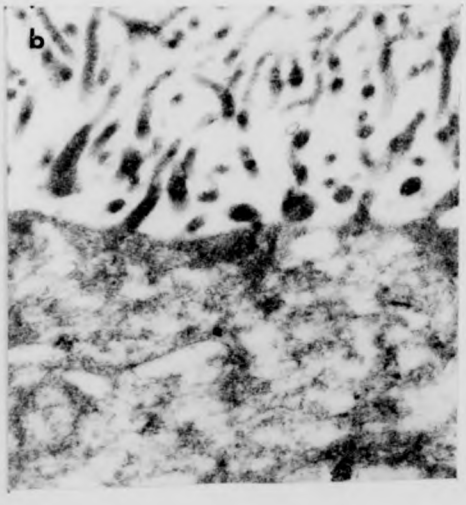
Print (b). 3 hours in Hanks' saline (X12,100). 11-day old cysticercoid.

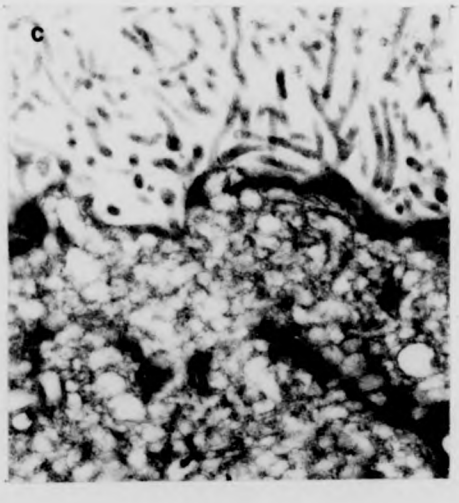
Print (c). 3 hours in Hoyle's saline (X25,050). 30-day old cysticercoid.











incubated in KPP, (c) a 30-day old cysticercoid that has been incubated in KRT and (d) another cysticercoid of the same age incubated in KPP. All of these prints, and those in Plate 3.3.3.3., are of the microvillar region and the immediately adjoining cytoplasm only. It was reasoned that the transport systems responsible for the transport of amino acids must be located in this region, and thus a study of the underlying tissues of the tegument was not relevant to this study. The integrity of the microvilli and apical plasmalemma of these saline-treated specimens was unimpaired when compared to the control (untreated) specimens, but on the surface of the KPP-treated specimens occasional 'blebbing' (b) was noted, and measurements of the width of KPP-treated microvilli showed that these structures had swelled slightly to 0.10 µm.

Plate 3.3.3.3. shows the appearance of a 30-day old cysticercoid after 5 hours incubation in KRT (a), an 11-day old specimen after 5 hours in Hanks' saline (b) and a 30-day old specimen after 5 hours in Hoyle's saline. These prints show little difference from the appropriate control cysticercoids. The width of the microvilli is comparable to the controls, and membrane integrity is retained.

Thus there is little ultrastructural evidence to suggest that incubation in any of the four salines has any effect on the integrity of the microvillar layer, apical plasmalemma or adjoining cytoplasm of the cysticercoid.

3.3.4. Uptake studies

The time course uptake of ^{14}C-cycloleucine by the cysticercoids is presented in Figure 3.3.4.1. From this graph it is apparent that the greatest quantity of cycloleucine is absorbed by cysticercoids that are maintained in KRT saline, followed by the KRP-maintained examples, then the Hanks'. The uptake of cycloleucine observed with cysticercoids that were maintained in Hoyle's saline was not sufficiently high to exceed that which would be accounted for by background radiation and the tube correction (see Section 2.4.).

3.3.5. Protein synthesis studies

The uptake and incorporation into protein of 0.1 mM ^{14}C-L-leucine is presented in Table 3.3.5.1. The highest rate of incorporation is supported by Hanks' saline, followed by KRT and Hoyle's salines equally, and finally KRP saline. These differences were statistically significant at the $p = 0.05$ level. The uptake rates are in the same, statistically valid (@ $p = 0.05$), rank order. It is interesting to note that the uptake of leucine differs from that of cycloleucine, the highest rate of absorption of leucine being supported by Hanks' saline, followed by Hoyle's and KRT equally, then KRP. Possible reasons for this difference will be considered in the Discussion (Section 3.4.).

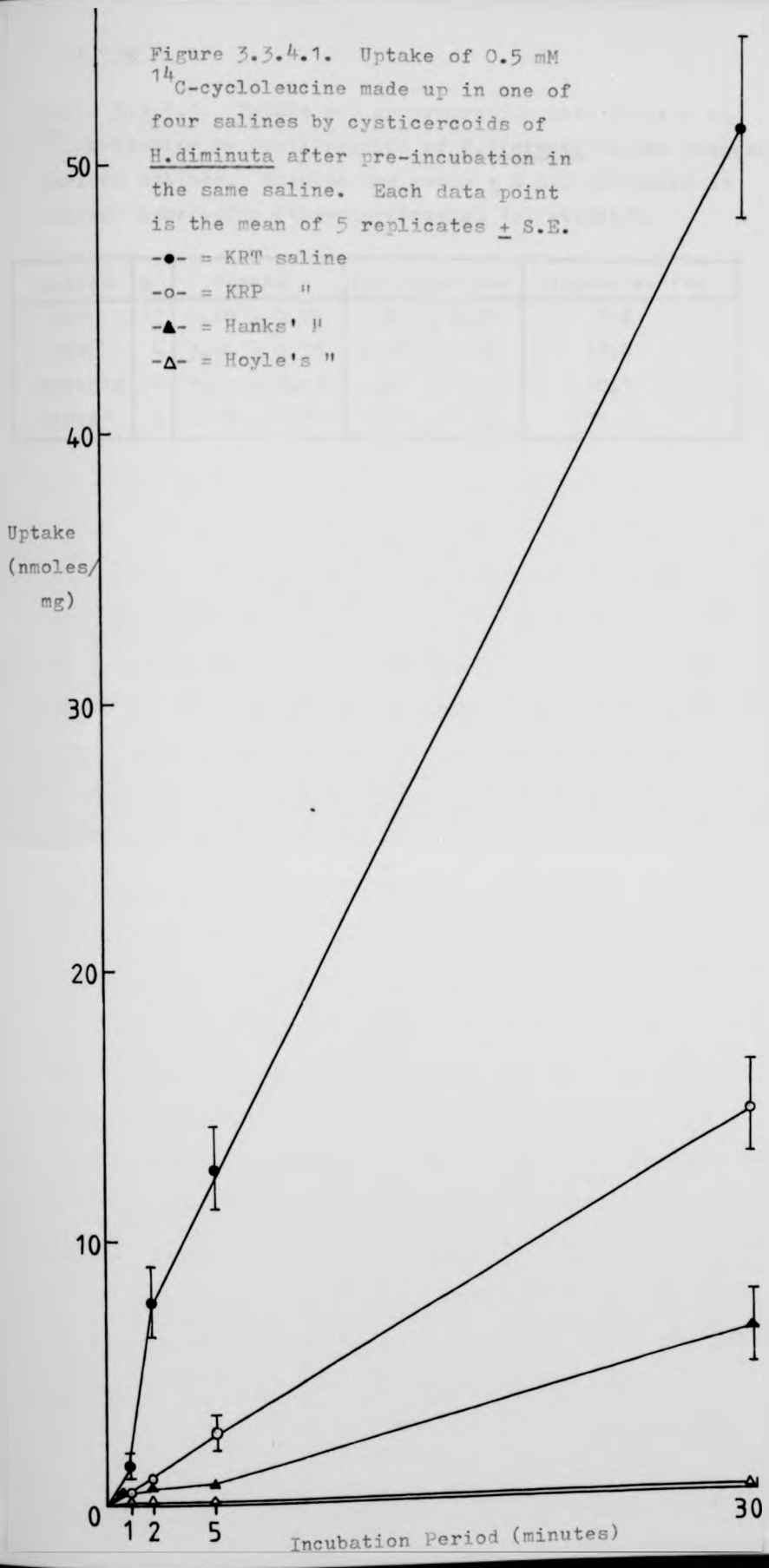


Figure 3.3.4.1. Uptake of 0.5 mM ^{14}C-cycloleucine made up in one of four salines by cysticercoids of H.diminuta after pre-incubation in the same saline. Each data point is the mean of 5 replicates ± S.E.

-●- = KRT saline

-o- = KRP "

-▲- = Hanks' "

-Δ- = Hoyle's "

Table 3.3.5.1. Uptake and incorporation into protein of ^{14}C-L-leucine by cysticercoids of H.diminuta in the presence of various salines. Results are means ± S.E., expressed in nmoles absorbed/mg ethanol-extracted dry weight/h.

Saline	n	Uptake	Incorporation	%Incorporation
KRT	10	6.87 ± 0.77	0.65 ± 0.07	8.6
KRP	4	3.96 ± 0.33	0.54 ± 0.02	12.0
HOYLE'S	4	8.05 ± 0.72	0.90 ± 0.09	10.1
HANKS'	3	10.16 ± 0.40	1.22 ± 0.10	10.7

3.4. Discussion

The experiments described do not permit an unequivocal answer to the question "which is the most suitable saline for the short term, *in vitro*, maintenance of *H.diminuta* cysticercoids". For example, if the results of the rat infection experiments (3.3.1.) alone are considered, one might conclude that Hoyle's is the most suitable saline. In contrast, the excystment experiments (3.3.2.), cycloleucine absorption tests (3.3.4.) and ultrastructural studies (3.3.3.) tend to suggest that KRT is the medium of choice. Finally, although the percentage incorporation rate of ^{14}C-L-leucine into protein does not vary with the saline employed, the actual quantity of ^{14}C-L-leucine-containing protein synthesised appears to be greatest in Hanks' saline. Regarding this latter finding, it is interesting to note that Weaver, Pratt, Hamnett and Jennings (1980) found that Hanks' saline was more effective in maintaining the biosynthesis of juvenile hormone from the isolated corpora allata of adult *T.molitor* than a variety of insect salines.

There have been few studies of the suitability of various salines for the *in vitro* maintenance of cestodes. Arme & Coates (1973) found that cysticercoids of *H.diminuta* survived in Hedon-Fleig's, Tyrode's or KRT saline for 24 hours, and that 18 out of 20 cysticercoids survived for 72 hours in KRT, as determined by their infectivity to rats. This latter finding could not be repeated in this study. From their results, Arme and Coates concluded that KRT saline was a suitable saline for *in*

vitro incubation. DeRycke (1975) found no significant differences in the infectivity to mice of H.microstoma cysticercoids that had been pre-incubated in either Ringer's saline or Eagle's Basal Medium for 1-6 days. Podesta, Stallard, Evans, Lussier, Jackson & Mettrick (1977) showed that the uptake of 5 mM glucose by adult H.diminuta over 2 minutes was inversely related to the pre-incubation period of the worms in one of either the 'Balanced Electrolyte Solution' of Podesta et al. (1977), KRT, or a hymenolepid cestode culture medium (see Appendices 1B, 1A and 1C respectively for compositions). For example the results obtained with KRT saline were significantly lower than those obtained with the other two media over the entire range of pre-incubation periods (1-6 hours). However, these results must be interpreted with caution due to the lengthy pre-incubation periods employed. Most workers concerned with cestode nutrient physiology have adopted the 30 minute pre-incubation period first employed by Read et al. (1963).

Concerning the results of Sections 3.3.4. and 3.3.5., the time course absorption of ^{14}C-cycloleucine is considered in Section 4.3., and of ^{14}C-L-leucine in Section 5.3.3. The total absorption of these two compounds from 0.2 mM solutions after 30 minutes incubation was 20.2 and 12.5 nmoles/mg dry weight respectively. The total amount of 0.5 mM ^{14}C-L-leucine that would be absorbed after 30 minutes has not been determined in this study, but it is clear that the two compounds are absorbed at different rates, and Section 5.3.5. suggests that although both compounds enter the cysticercoid through the 'A(alanine)'

and 'L(leucine)' loci postulated for the cysticercoid, leucine preferentially enters through the latter locus and also utilises the 'Phenylalanine' locus which is apparently not available to cycloleucine. Although the involvement of ions in the direct transport of amino acids by cestodes is unclear (see Introduction, Section 1), it is quite possible that they may have an indirect involvement. Lussier, Podesta & Mettrick (1978) have found that the uptake of amino acids by adult H.diminuta can be altered by adding ATP to the incubation medium, and several ATPases have been isolated from preparations of the brush border membrane of this worm (see Pappas, 1983b, for discussion).For example, Rahman, Mettrick & Podesta (1981) have isolated a ouabain-insensitive ATPase from this membrane, the activity of which was stimulated by Mg^{2+} and Na^{+}, and inhibited by Ca^{2+}. Although this enzyme appears to function in volume regulation one can assume that other ATPases may well have an indirect connection with amino acid transport, and their activity may be controlled by the available levels of certain cations. Thus certain salines may have an effect on the transport of certain amino acids depending on the ion-dependence of the transport loci which they utilise. In the Ehrlich cell, the L system, which is primarily used by leucine, is Na^{+}-independent, and the A system, which is primarily used by cycloleucine, is Na^{+}-dependent. Systems (loci) very similar to these also appear to exist in the cysticercoid of H.diminuta (Section 5.3.5.). Although the ion-dependency of these cysticercoid loci is not yet known, it is possible that the differing effects of the salines used in this study upon the uptake of leucine and cycloleucine may be due

to such a dependence.

A critical finding of the current study was that all four salines caused few changes at the ultrastructural level. Contrary findings have been noted in studies concerning S.mansoni. Thus, Wilson & Barnes (1974), found that although S.mansoni incubated for 5 hours in Hanks' saline survived, ultrastructural damage, in the form of vacuolations of the tegument and the tegumental cell bodies, was noticeable after 30 minutes incubation. After 4 hours incubation, the electron density of the tegument-cytoplasm had increased when compared with untreated worms, a considerable extension of surface invaginations had occured, and the perinuclear cytoplasm within the tegumental cell bodies had become extensively vacuolated. Shaw & Erasmus (1977) found that the tegument of S.mansoni after treatment with the drug Astiban (Roche), showed, amongst other changes, an increased electron density. The process of tegumental disruption attributed to the action of this drug has many similarities to that brought about by other adverse conditions, such as hypertonic media, and exposure to immune serum (Hockley, 1973).

It is clear from the foregoing discussion that none of the four salines is ideal from all points of view. Until a complete analysis of Tenebrio haemolymph is available, it has been concluded that KRT saline is adequate for the maintenance of cysticercoids in vitro for short time periods. This saline also has the advantage of being the saline used in most investigations of adult H.diminuta physiology, and thus its adoption for this study may facilitate comparison of data.

4. UPTAKE OF CYCLOLEUCINE - PRELIMINARY STUDY AND AGE-DEPENDENCE

4.1. Introduction

As stated in the Introduction (Section 1) there is good evidence that both the adults and cysticercoids of H.diminuta are permeable to low molecular weight nutrients. This section examines whether the age-dependent morphological changes that occur during cysticercoid development (see Fig. 1.2. and Section 2.4.7.) are associated with physiological changes in the pattern of nutrition.

In this study, the uptake of ^{14}C-cycloleucine has been studied using both stage III and stage V cysticercoids. The section comprises two parts. In the first, the characteristics of ^{14}C-cycloleucine absorption by stage V cysticercoids are established, commencing with an examination of time-dependent absorption at selected substrate concentrations (Section 4.3.). From this data, the initial rate period (i.e. the time period over which cycloleucine transport remains largely uni-directional) can be determined for the kinetic studies that comprise Section 4.4. The second part of the section examines possible changes in the nutritional status of the cysticercoid as it ages. These are the subject of Section 4.5., which consists of time course and kinetic studies. The section concludes with a discussion (Section 4.6.).

4.2. Theoretical Background

4.2.1. Calculation of concentration ratios

The extent to which cycloleucine is accumulated can be determined from the time-dependent absorption data by calculating the 'concentration ratio', which is a measure of the concentration of the compound within the parasite compared to its concentration in the ambient medium. To quantify concentration, a knowledge of the amount of water within the cysticercoid is required, and for the purposes of this study, the mean dry weight : fresh weight ratio of 81% provided for stage V cysticercoids of H.diminuta by Arme & Coates (1973) will be used. It is possible that this ratio may change with cysticercoid development, but the use of this single extreme dry weight value will allow general conclusions to be drawn as to the ability of cysticercoids of all ages to accumulate cycloleucine. It is also assumed that cycloleucine is not compartmentalised within the cysticercoid.

4.2.2. Accuracy of K_t and V_{max} values

Throughout this study, the kinetic parameters K_t and V_{max} have been derived from the graphical method of Lineweaver & Burk (1934)(see Figure 1.6.2.). This method has two major disadvantages. Firstly, it tends to obscure any deviations from Michaelis-Menten kinetics, and secondly, it is

considered to be the least accurate method for the determination of kinetic constants (Dowd & Riggs, 1965). Regarding the second criticism, since small errors in one or two points can dramatically affect the intercepts of kinetic plots, calculations involving intercepts are extremely sensitive to experimental variability. Comparable fluctuations disturb the slopes of such plots in a more trivial manner. Thus linear transformations involving the calculation of kinetic constants from the slopes of kinetic plots are to be preferred over those using intercepts, and are more likely to reflect the true properties of the system under study. Suitable linear transformations that do not involve the use of intercepts are those of Eadie (Eadie, 1942), in which v is plotted against $v/[S]$, and the slope of the plot = $-K_t$, and Woolf (Dowd & Riggs, 1965), in which $[S]/v$ is plotted against $[S]$, and the slope of the plot = $1/V_{max}$. Eadie plots have the additional advantage of being most sensitive to systematic deviations from Michaelis-Menten kinetics, and have been used by Starling & Fisher (1975) to examine the uptake of hexose sugars by Moniliformis dubius. When the data concerning ^{14}C-galactose and ^{14}C-fructose uptake by this acanthocephalan were analysed by the method of Lineweaver & Burk, deviations from linearity were not apparent either from a visual inspection of the plots or calculation of the correlation coefficient from least-squares regression analysis which exceeded 0.99. However, deviations from Michaelis-Menten kinetics were readily apparent when either Eadie or Woolf plots were used. At the present time, these linear transformations have been superseded by such non-linear

least squares curve fitting procedures as that of Atkins & Gardner (1977), which are considered to offer considerable advantages over the previous linear curve fitting procedures (Markus, Hess, Ottaway & Cornish-Bowden, 1976). The method of Atkins & Gardner allows values of K_t, V_{max} and K_D to be generated directly from kinetic data, and by using this transformation Lussier et al. (1982) disclosed the presence of a hitherto unsuspected diffusion component in the uptake of methionine by adult H.diminuta. Despite the criticisms against its use, however, as this study is largely a comparative one with the adult worm, the method of Lineweaver & Burk will be employed as this has been the method of choice in most previous studies concerning adult H.diminuta.

Reference to Figure 1.6.2. illustrates the method used to calculate K_t and V_{max}. The line in all cases has been fitted by a method of least squares, but this line of best fit will only give a best estimate of the intercept on the y-axis and the value of the gradient. The degree of uncertainty in this estimate can be quantified by calcualting the Standard Error of Estimate (S.E.E.) for each plot from the formula:-

$$S_{x \text{ (or } y)} = \sigma_{x \text{ (or } y)} \sqrt{1 - r^2}$$

Where σ = the appropriate standard deviate of the experimental value and r = the product moment correlation coefficient. By calculating c (the intercept of the line on the y-axis) $\pm$ S.E.E., the variation in the values of K_t and V_{max} can be determined.

In 95% of the cases, the actual values will lie between plus or minus 2 x S.E.E. (Moroney, 1978). The ranges of K_t and V_{max} (when given in both this section and Section 5) are always for these 95% probability limits. It is not necessary to calculate the variation in the gradient, within the limits calculated for the y-intercept, as this will still intercept the y-axis at the same point whatever its value (see Figure 4.2.2.1.).

4.2.3. Separation of passive and mediated uptake

If a combination of transport processes is apparent from a Michaelis-Menten plot of uptake data (see line C_1, Figure 1.6.1.), the two components may be separated by one of three methods. However, it must be stressed that a linear relationship between the rate of substrate absorption and substrate concentration does not necessarily imply that diffusion is taking place. It is possible that this linear portion of the kinetic plot represents a mediated transport mechanism which saturates at concentrations in excess of those employed in the experiment. To determine whether diffusion really exists, the method of choice is to examine the uptake of a fixed concentration of the chosen compound in the presence of increasing concentrations of unlabelled inhibitor. By plotting uptake as the y-axis against inhibitor concentration as the x-axis a plot will be obtained in the form of Figure 4.2.3.1. If the inhibitor enters the cell through all of the same loci that the test compound uses, at a certain I:S ratio the uptake of the test

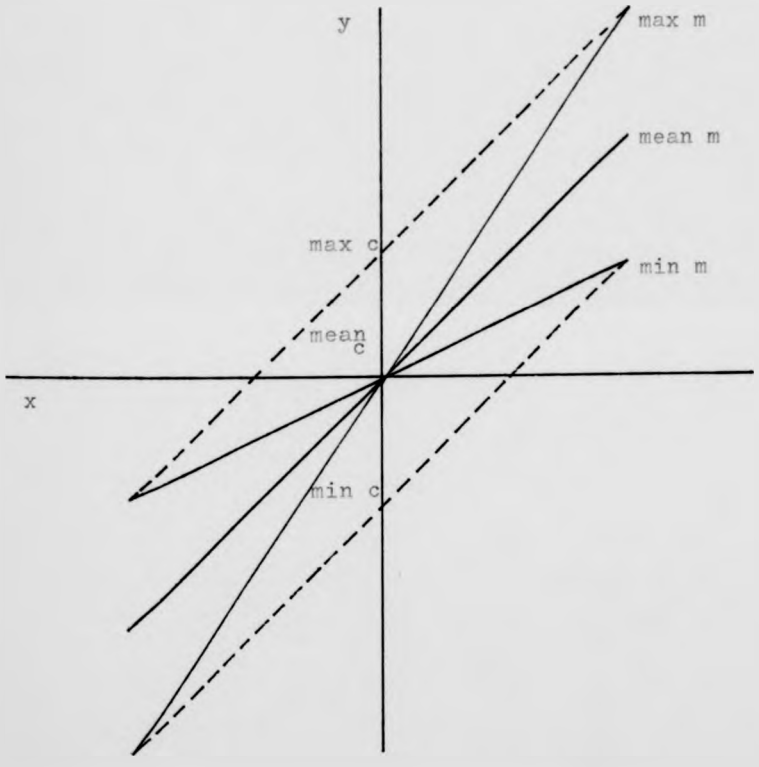


Figure 4.2.2.1. Variations in the values of the constants m and c (see text for details)

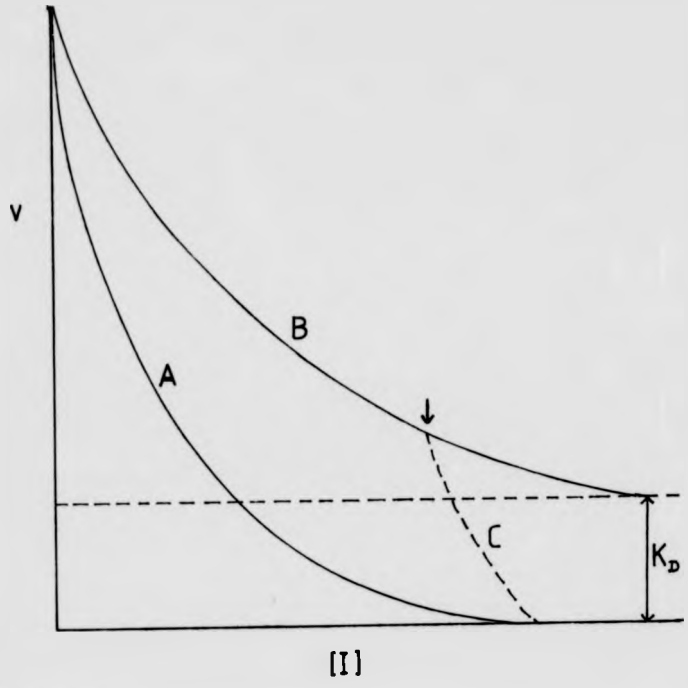


Figure 4.2.3.1 Line A represents the uptake of the test compound in the presence of an inhibitor which blocks all of the uptake loci used by the test compound; uptake occurs by mediated transport alone. Line B represents the situation when the uptake of the test compound occurs by a combination of passive and mediated transport. However, the addition of suitable inhibitors (at point marked by arrow) may reveal the diffusion component of transport to represent mediated transport of the test compound through uptake loci not blocked by the first inhibitor (line C).

compound should be completely supressed. If it is not, additional inhibitors should be added which will block the entry of the test compound through the uptake loci not blocked by the first inhibitor. If, when the full range of possible inhibitors have been tried at high I:S ratios, the uptake of the test compound is still apparent, it is likely that the portion of uptake remaining is by passive diffusion alone. Besides being a useful method for the separation of passive and mediated transport, this method can also be used to test for the presence of a diffusion component of transport even when the form of the Michaelis-Menten plot would tend to mitigate against such an event, and in addition it has been extensively used to determine the relative interactions of groups of structurally-similar compounds with their transport loci (see, for example, the study of Woodward & Read (1969) concerning the transport of histidine through two distinct systems in the adult H.diminuta).

A value of K_D, the diffusion coefficient, can be calculated directly from the linear portion of a Michaelis-Menten type plot by dividing the increase in uptake over the linear portion of the plot by the increase in substrate concentration over the same portion. By dividing this value by the substate concentration at each data point, and subtracting the value so obtained from each data point, passive and mediated transport may be separated. If the value of K_D obtained by this method compares to the value obtained by the method outlined above, it is most likely that the linear portion of the Michaelis-Menten plot does represent

uptake by passive diffusion. A more refined method for the separation of passive and mediated transport from kinetic data is that of Akedo & Christensen (1962). The net inward migration of a solute, A, can be described as the sum of two components, Y, the mediated flux, and K_p ($[S]_1$ - $[S]_2$), the diffusive flux, where K_p is the permeability coefficient with dimensions of min^{-1}:-

$$\frac{d[A]}{dt} = Y + K_p ([S]_1 - [S]_2) \qquad -(1)$$

Which, on integration, becomes:-

$$\frac{[A]_2}{[A]_1} = Y (1 - e^{-K_p t}) \cdot \frac{1}{[A]_1} + (1 - e^{-K_p t}) \qquad -(2)$$

This equation is in the form y = mx + c. At high solute concentrations the value of Y will be essentially constant, so by plotting $[A]_2/[A]_1$ (uptake at high solute concentrations) against $1/[A]_1$ (the solute concentration), the intercept of the line on the y-axis will give $(1 - e^{-K_p t})$. Hence, K_p can be found and inserted into equation (2), from which Y can be calculated. The third method is that of Atkins & Gardner (1977), which has already been mentioned (Section 4.2.2.).

4.2.4. A note on experimental design

All of the data to be presented in Sections 4 and 5 have

been derived from incubating pools of 25 cysticercoids of known age in selected concentrations of ^{14}C-labelled amino acids for specified incubation periods. In all graphs, uptake rate is designated by v, which has dimensions of μmoles/g/h (unless otherwise specified), concentration of test solute (substrate) by $[S]$, with dimensions of mM and inhibitor solute by I, also with dimensions of mM.

4.3. Time Course Studies

Figure 4.3.1. suggests that cysticercoids rapidly absorb ^{14}C-cycloleucine from a 0.5 mM solution. Uptake appears to decline after about 30 minutes, and a steady state is reached after about 45 minutes. This steady state may represent equal rates of influx and efflux, cell death, substrate depeletion, changes in pH or oxygen tension or a combination of these factors. Concentration ratios exceeding unity are rapidly built up in cysticercoids incubated in 0.5 mM ^{14}C-cycloleucine (Figure 4.3.2.) and after 10 minutes incubation in 0.1, 0.5 or 1.0 mM ^{14}C-cycloleucine solutions, concentration ratios of 6.8, 6.4 and 5.4 respectively are achieved.

Arme & Coates (1973) employed 2 minute incubation periods for their kinetic studies. Figure 4.3.3. shows the uptake of 0.1 mM and 1.0 mM ^{14}C-cycloleucine over 10 minutes. The linear relationship between cycloleucine uptake and incubation time would tend to suggest that uptake is unidirectional over this time period and for these substrate concentrations. However, further experiments tended to show a slight deviation from linearity after 5 minutes particularly at the lower concentrations (Figure 4.3.4.). A 5 minute incubation time was thus adopted for all further kinetic studies involving cycloleucine.

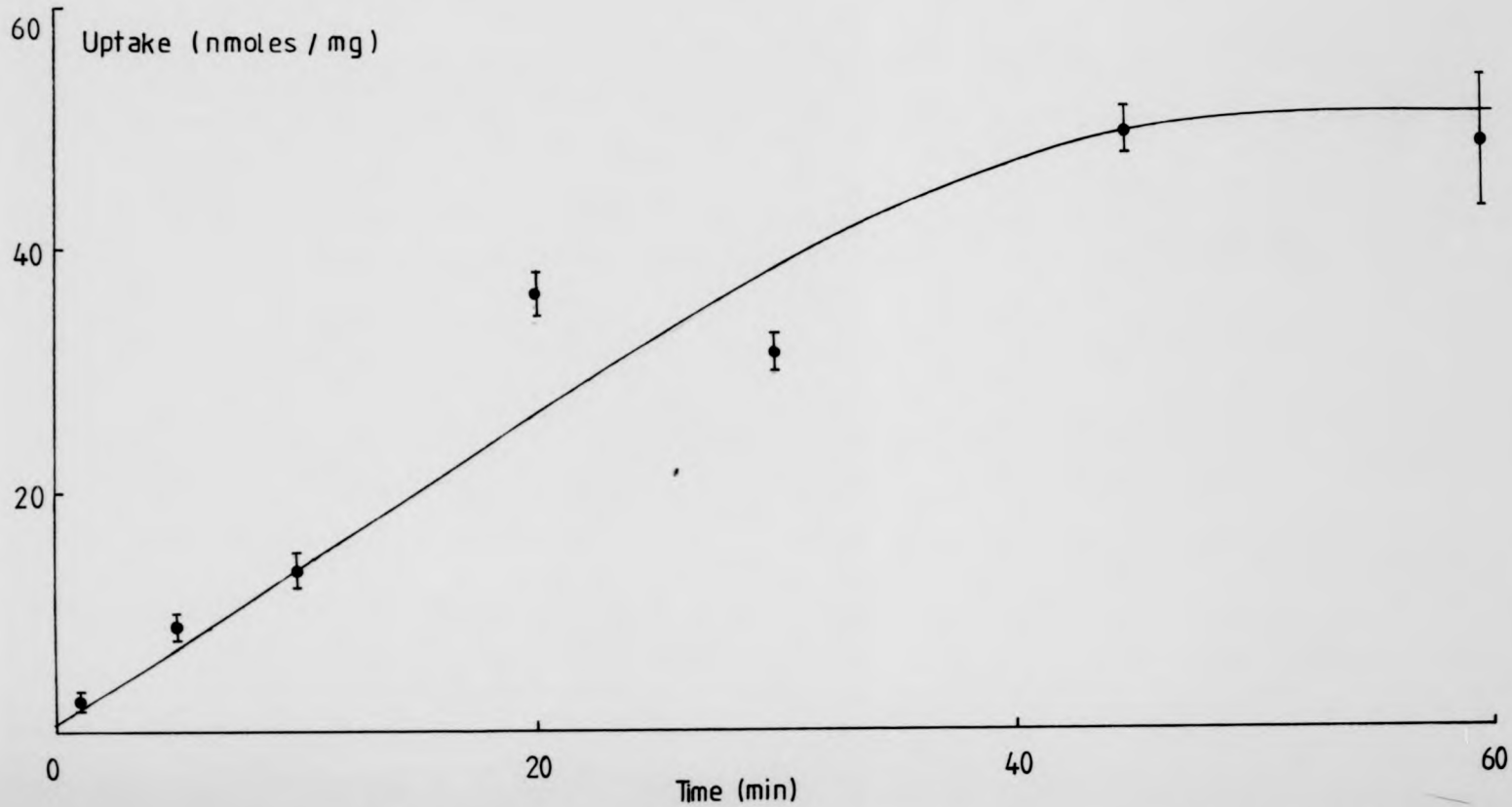


Figure 4.3.1. Uptake of 0.5 mM ^{14}C-cycloleucine with respect to incubation time. Each data point is the mean of 5 replicates ± S.E.

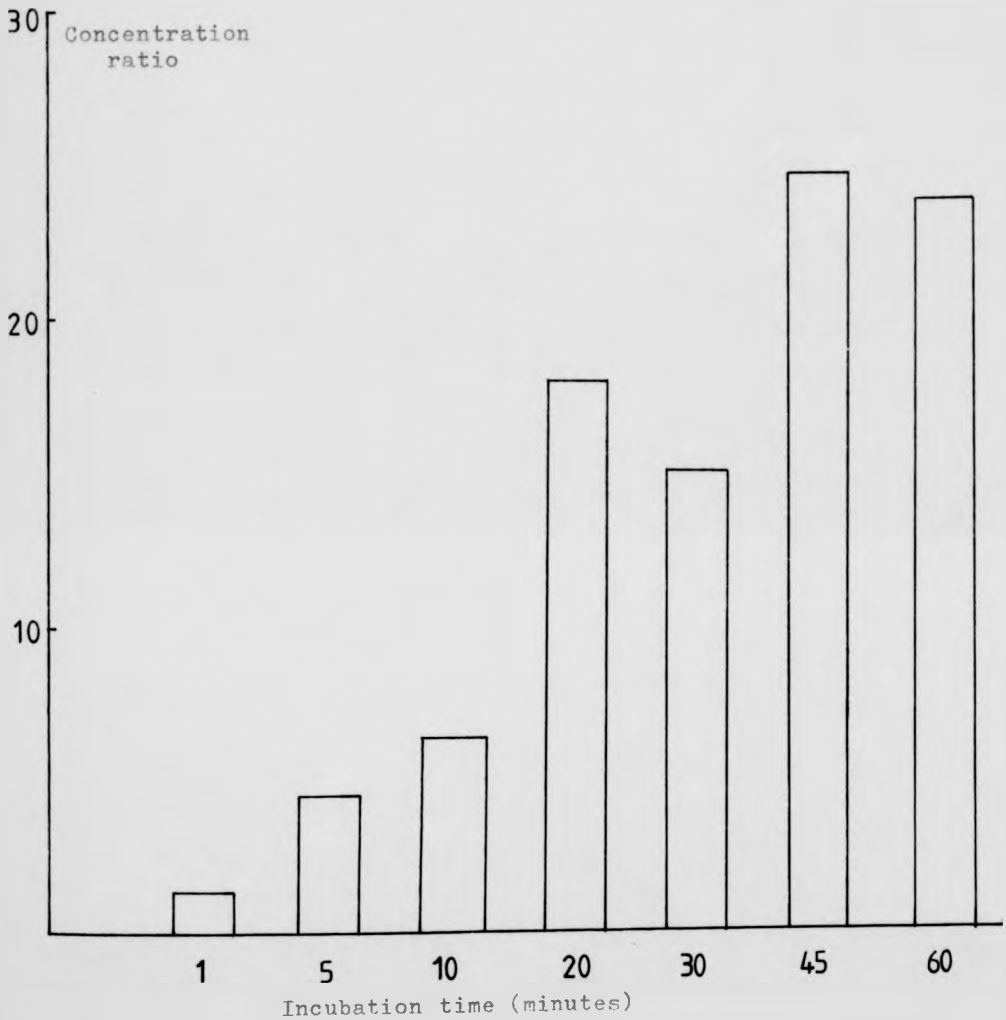


Figure 4.3.2. Concentration ratios achieved after incubation of cysticercoids in 0.5 mM cycloleucine for varying time periods (see text for details).

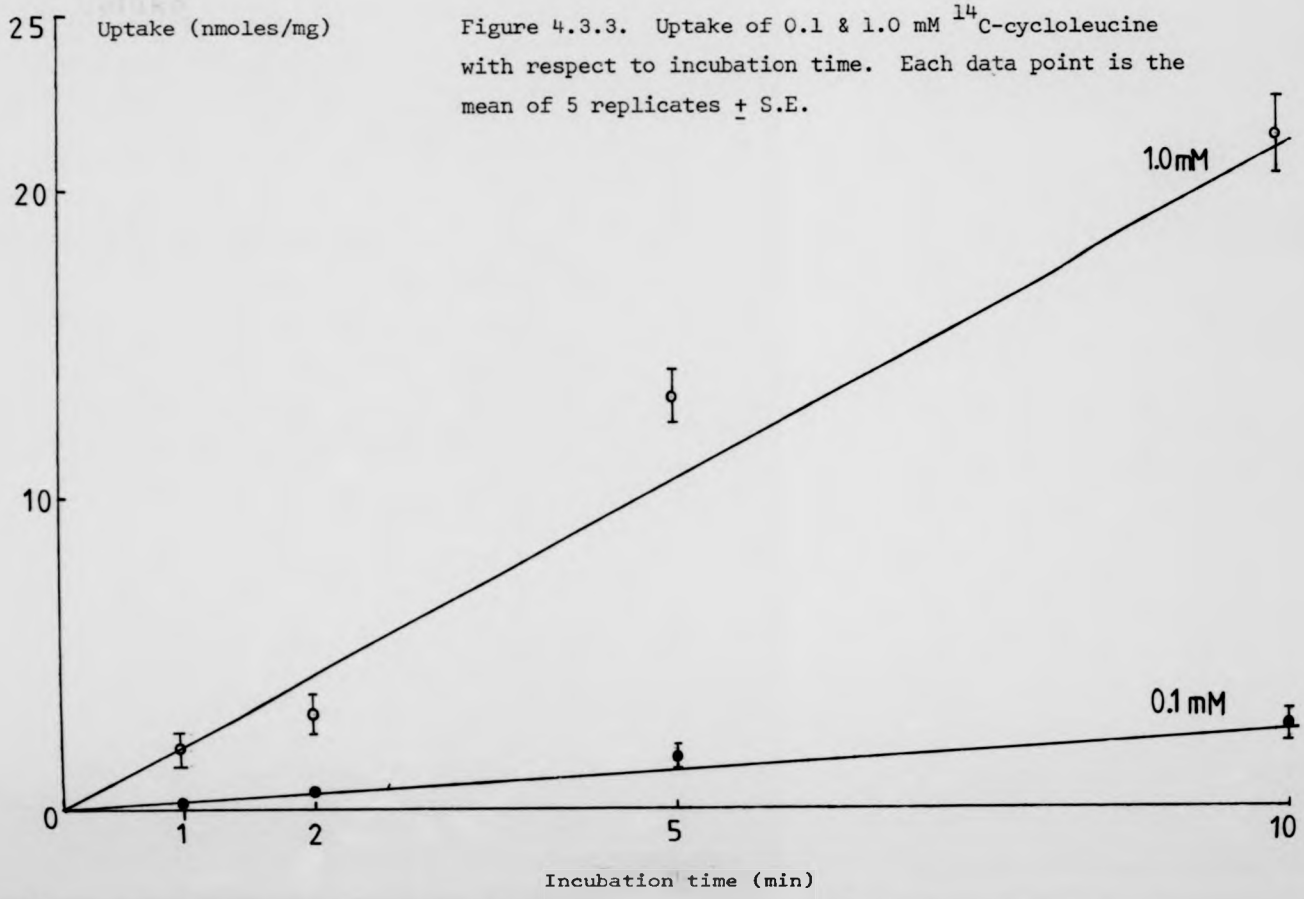


Figure 4.3.3. Uptake of 0.1 & 1.0 mM ^{14}C-cycloleucine with respect to incubation time. Each data point is the mean of 5 replicates ± S.E.

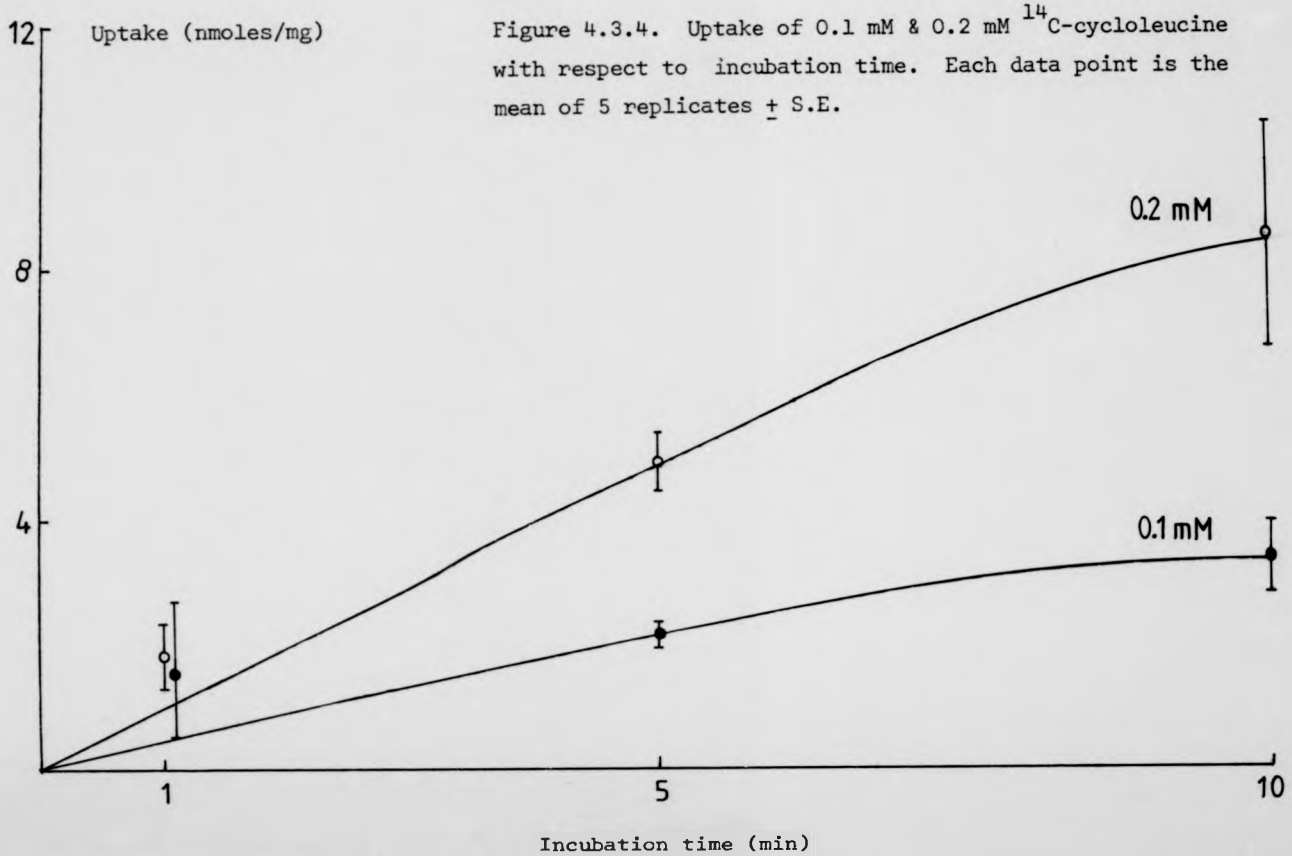


Figure 4.3.4. Uptake of 0.1 mM & 0.2 mM ^{14}C-cycloleucine with respect to incubation time. Each data point is the mean of 5 replicates ± S.E.

4.4. Kinetic Studies with Stage V Cysticercoids

The rates of uptake obtained when 14 or 15 day old cysticercoids are incubated for five minutes in ^{14}C-cycloleucine solutions of concentrations between 0.1 and 5.0 mM are shown in Figure 4.4.1. It can be seen that uptake is non-linear with respect to concentration, following Michaelis-Menten type saturation kinetics. Saturation appears to occur at a substrate concentration of about 2 mM, and thereafter there is little increase in the rate of uptake up to 5 mM. This would suggest that a diffusion component of cycloleucine uptake, if it exists, is very small. Diffusion is further examined later in this section. When the data of Figure 4.4.1. is plotted by the method of Lineweaver & Burk, a staight line is obtained (Figure 4.4.2.). From this graph, a mean value for V_{max} of 177 µmoles/g/h (range 127-292 µmoles/g/h), and a mean value for K_t of 0.12 mM (range 0.09-0.20 mM) can be obtained.

When these values are compared to those obtained with adult H.diminuta by Harris & Read (1968), a similar V_{max} value of 170 µmoles/g/h is found, but a slightly different K_t value of 0.25 mM. This may be due to the concentration range, and the increment between concentrations (0.05, 0.1, 0.2, 0.5, 1.0 mM), used by these authors. It is notable that the concentrations double as they increase. This form of increase is considered preferable by Dixon & Webb (1964) when a Lineweaver-Burk plot is to be employed. The results of eight experiments involving cysticercoids of various ages that

have been incubated for 5 minutes in 0.1-1.0 mM ^{14}C-cycloleucine are shown as Michaelis-Menten type plots in Figure 4.4.3.1.-4.4.3.8., and Lineweaver-Burk plots in Figure 4.4.4.1.-4.4.4.8. Each data point is the mean of 5 replicate experiments (error bars have been omitted for clarity). The values of K_t and V_{max} for each of the eight experiments are given in Table 4.4.1. The Michaelis-Menten plots support the view that ^{14}C-cycloleucine uptake is non-linear with respect to concentration. Furthermore, the kinetic data suggest that when similar concentration ranges are employed, the values of K_t and V_{max} that can be obtained from both the adult and cysticercoid of H.diminuta are strikingly similar. The concentration range 0.1-1.0 mM will henceforth be employed for all ensuing studies involving the uptake of ^{14}C-cycloleucine, in order that comparisons can be made between the cysticercoid and adult worm.

Harris & Read (1968) found α-AIB to be a potent inhibitor of ^{14}C-cycloleucine uptake by the adult H.diminuta. In five replicate experiments, a mean uptake of 100 μmoles/g/h is observed when pools of 25 cysticercoids are incubated in 0.2 mM ^{14}C-cycloleucine for 5 minutes. In the presence of 2 mM unlabelled α-AIB, the mean uptake fell to 16 μmoles/g/h, representing an inhibition of 84%. This result would suggest that α-AIB is a highly effective inhibitor of ^{14}C-cycloleucine uptake, and is thus a suitable compound to use in an experiment designed to evaluate whether any component of the uptake of ^{14}C-cycloleucine by the cysticercoid occurs by passive diffusion. The theoretical basis of such an experiment is outlined in

Section 4.2.3., and Figure 4.4.5. shows the results of experiments when 26 day-old cysticercoids are incubated for 5 minutes in 0.1 mM ^{14}C-cycloleucine in the presence of 0.05, 0.1, 1.00, 5.00 or 7.50 mM unlabelled α-AIB. This represents substrate: inhibitor ratios of 0.5, 1.0, 10, 50 and 75:1 respectively. From the graph it can be seen that α-AIB apparently inhibits completely the uptake of ^{14}C-cycloleucine at an inhibitor:substrate ratio of 75:1, and thus it seems likely that little, if any, cycloleucine enters the 26 day-old cysticercoid by passive diffusion.

Thus, cysticercoids of a variety of ages appear to be capable of absorbing and accumulating ^{14}C-cycloleucine by mediated processes. From the data of kinetic experiments, values of K_t and V_{max} can be readily determined. Cycloleucine therefore appears to be suitable for use as a physiological 'probe' with which to chart any changes in nutritional status that may occur as the cysticercoid ages.

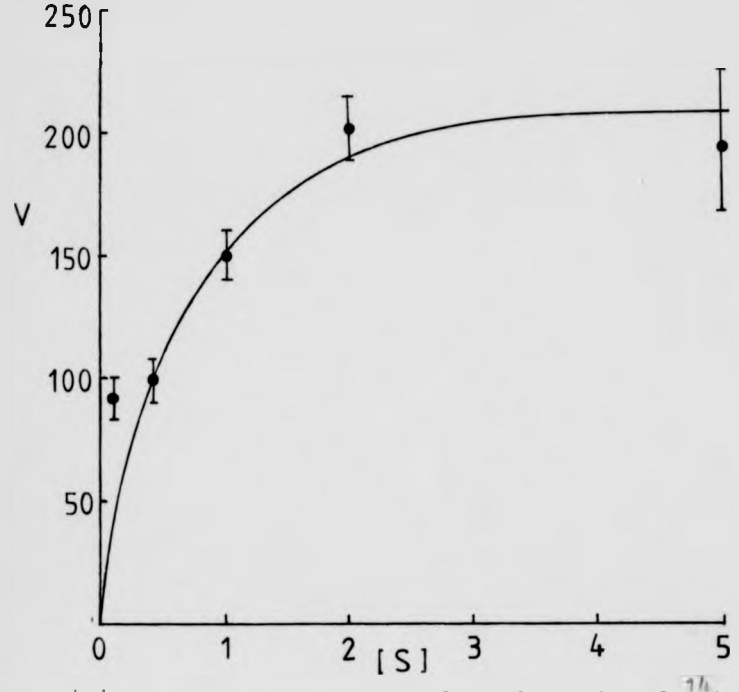


Figure 4.4.1. Michaelis-Menten plot of uptake of ^{14}C-cycloleucine. Each data point is the mean of at least 17 replicates ± S.E.

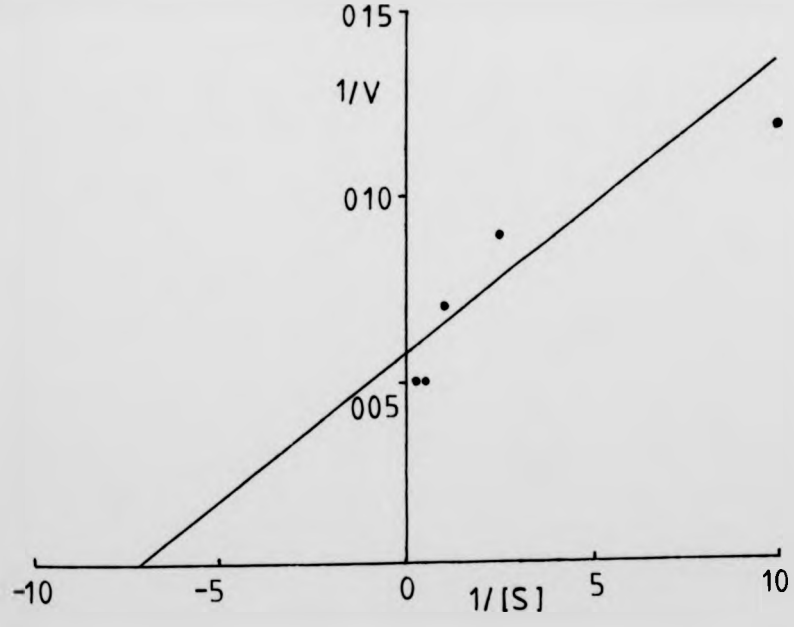
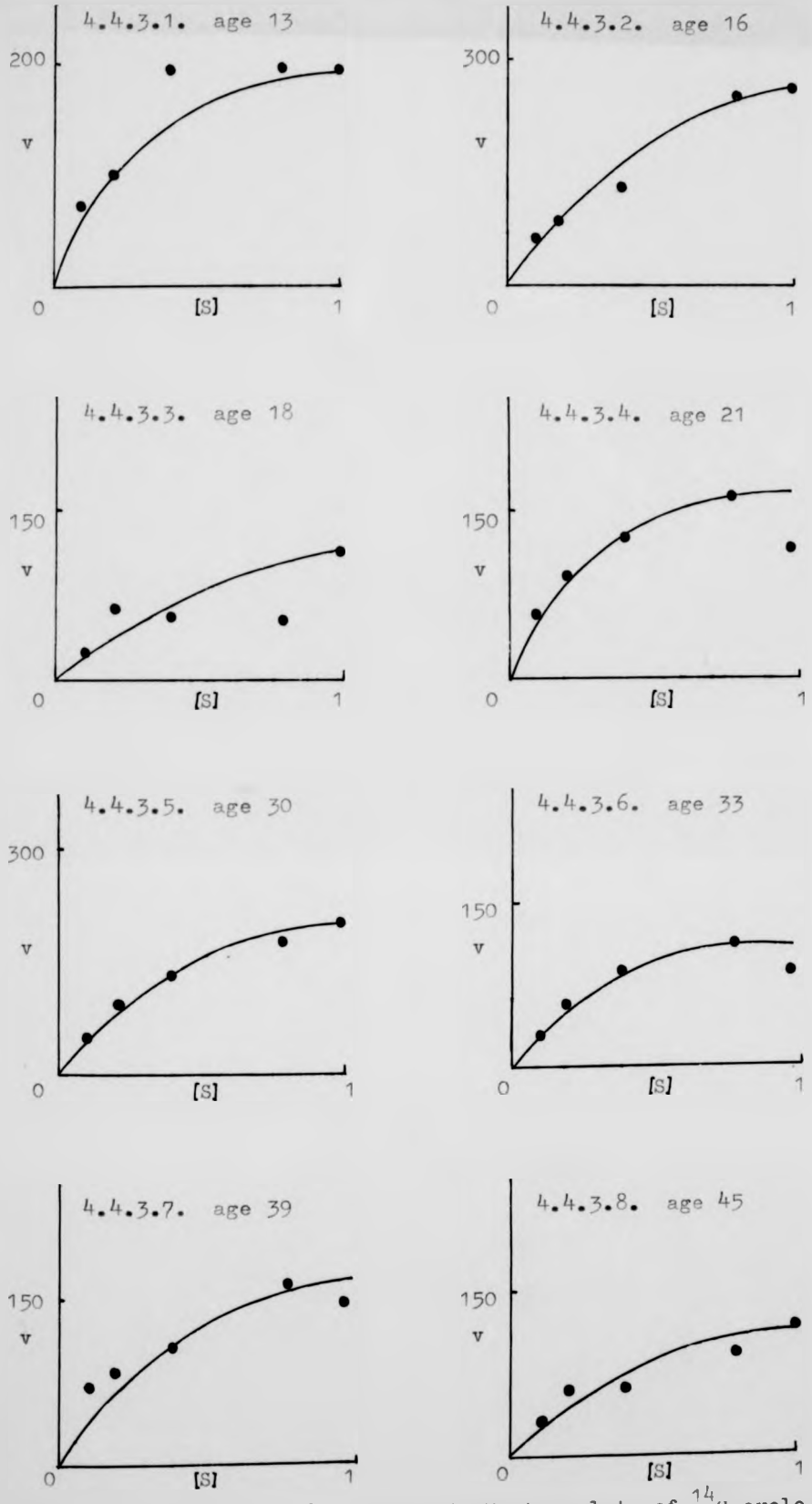
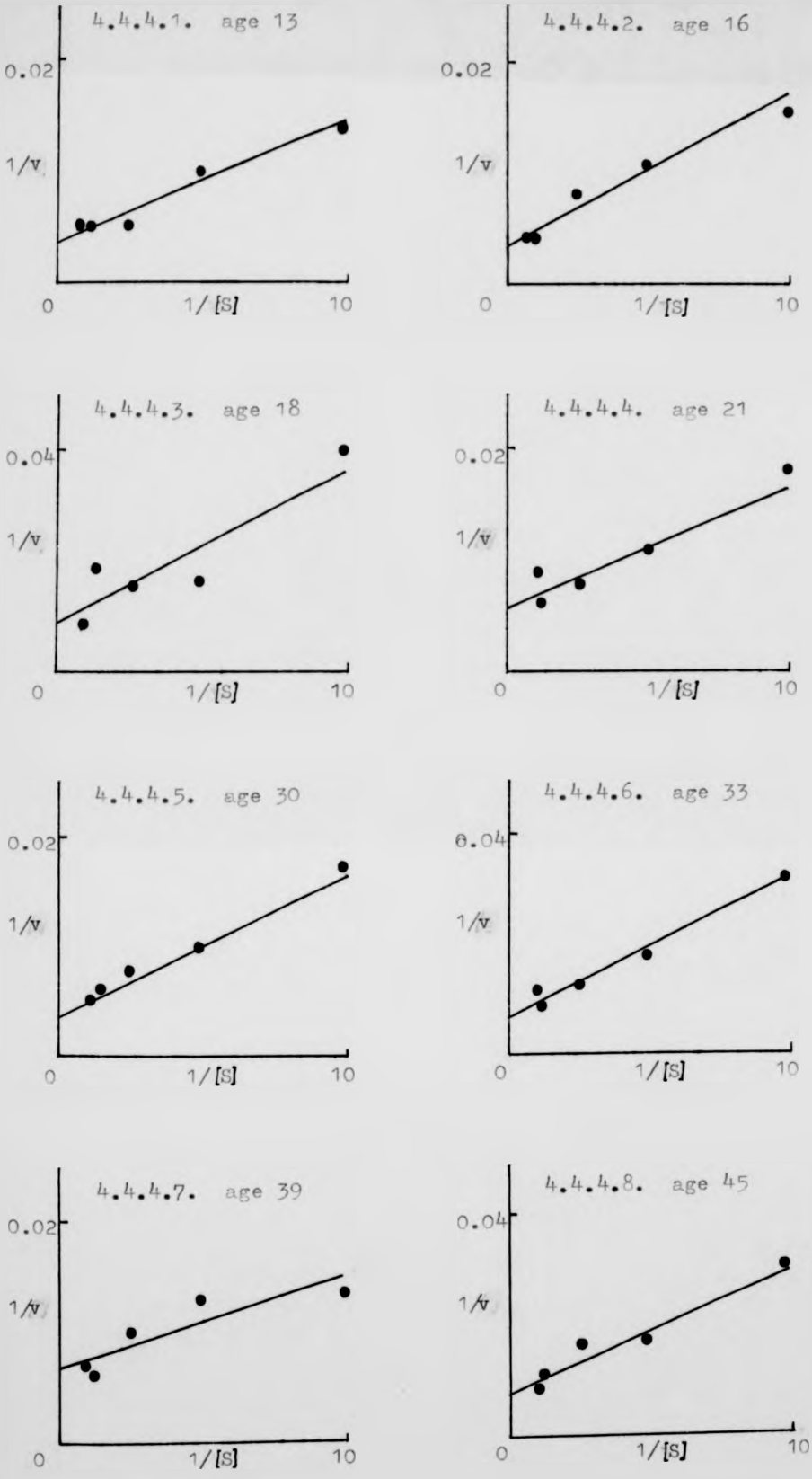


Figure 4.4.2. Lineweaver-Burk plot of Figure 4.4.1.



Figures 4.4.3.1.-4.4.3.8. Michaelis-Menten plots of ^{14}C-cycloleucine uptake by cysticercoids of various ages.



Figures 4.4.4.1.-4.4.4.8. Lineweaver-Burk plots of Figures 4.4.3.1.-4.4.3.8.

Table 4.4.1. K_t and V_{max} values derived from data presented in graphs 4.4.4.1. - 4.4.4.8.

Parasite age	K_t (mM)	V_{max} (μmoles/g/h)
13	0.30	281
16	0.40	298
18	0.32	114
21	0.21	176
30	0.31	241
33	0.34	142
39	0.13	151
45	0.27	121
Mean ± S.E.	0.29 ± 0.03	191 ± 26

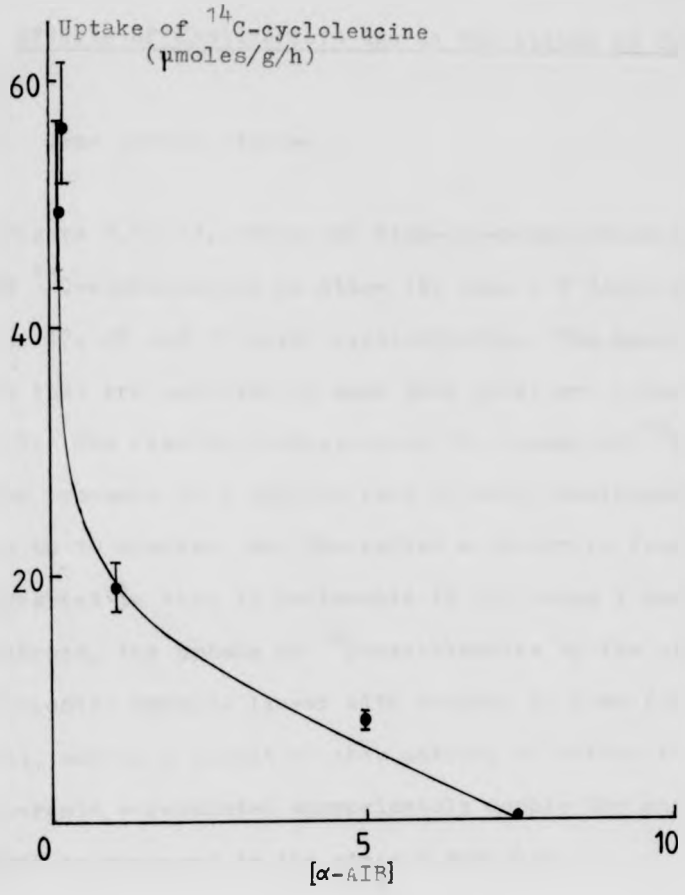


Figure 4.4.5. Uptake of 0.1 mM ^{14}C-cycloleucine in the presence of increasing concentrations of unlabelled α-AIB. Each point is the mean of at least 14 replicates ± S.E.

4.5. Effects of Cysticercoid age on the Uptake of Cycloleucine

4.5.1. Time course studies

Figure 4.5.1.1. shows the time-dependent absorption of 0.1 mM ^{14}C-cycloleucine by stage III (age = 9 days) and stage V (ages = 17, 29 and 39 days) cysticercoids. The concentration ratios that are achieved at each data point are listed in Table 4.5.1.1. The results indicate that the uptake of ^{14}C-cycloleucine proceeds at a similar rate in both developmental stages for up to 10 minutes, but thereafter a deviation from linearity with respect to time is noticeable in the stage V examples. In contrast, the uptake of ^{14}C-cycloleucine by the stage III cysticercoids remains linear with respect to time for up to 30 minutes, and as a result of this pattern of uptake the stage III cysticercoid accumulates approximately double the amount of this compared as compared to the stage V examples.

4.5.2. Kinetic studies with cysticercoids of different ages

Michaelis-Menten and Lineweaver-Burk plots of the uptake of ^{14}C-cycloleucine by stage III (9 day-old) cysticercoids are presented in Figures 4.5.2.1. and 4.5.2.2. respectively. From Figure 4.5.2.2., a mean K_t value of 0.21 mM (range = 0.13-0.44 mM) and a mean V_{max} value of 173 µmoles/g/h (range = 113-376 µmoles/g/h) can be calculated. These values compare closely to those obtained with both the stage V cysticercoid and the adult worm.

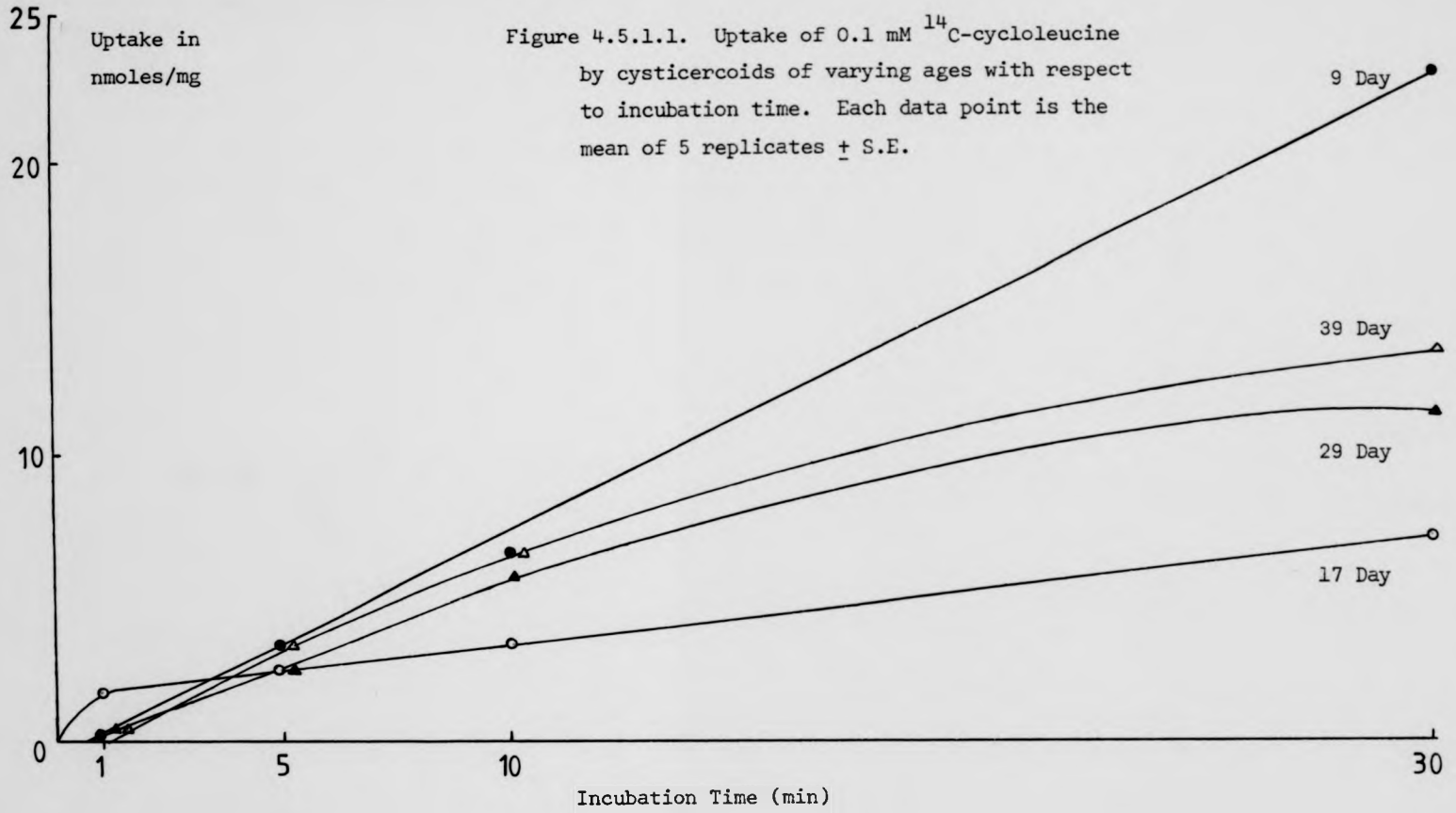


Figure 4.5.1.1. Uptake of 0.1 mM ^{14}C-cycloleucine by cysticercoids of varying ages with respect to incubation time. Each data point is the mean of 5 replicates ± S.E.

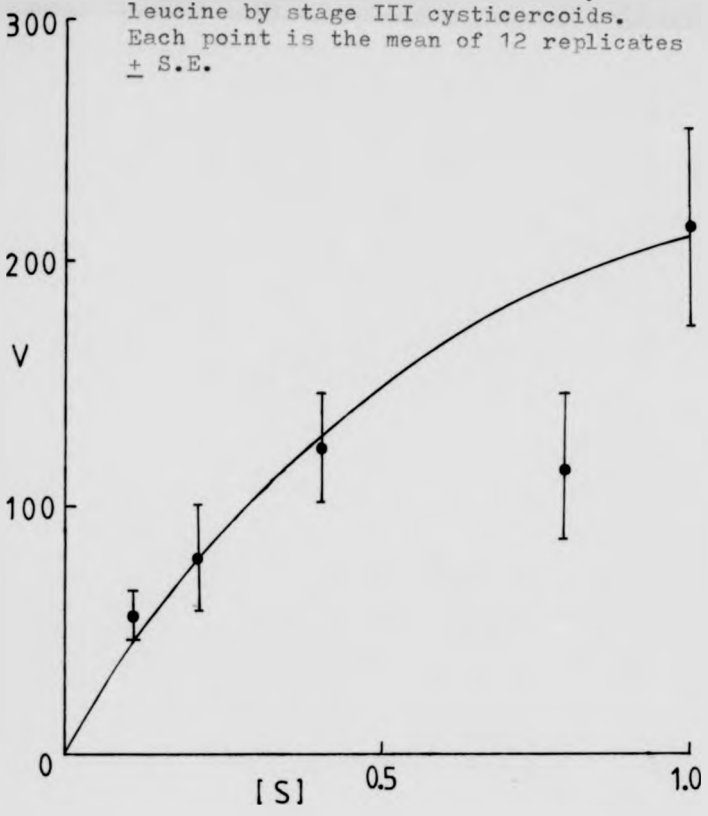
Table 4.5.1.1. Concentration ratios of uptake data from Figure 4.5.1.1.

Cysticercoid age (days)	Time (minutes)			
	1	5	10	30
9	0	8.4	17.1	57.5
17	4.0	5.5	8.5	17.7
29	0.9	6.2	13.4	28.9
39	1.2	8.6	16.7	34.4

The results of 38 experiments designed to determine the values of K_t and V_{max} from stage V cysticercoids aged between 12 and 47 days are presented as Figures 4.5.2.3. and 4.5.2.4. The degree of correlation between the age of the cysticercoids and K_t or V_{max} has been examined by the calculation of the product moment correlation coefficient, r. Although the line of best fit through the data points of Figure 4.5.2.3. suggests that the value of K_t falls from 0.27 mM to 0.20 mM, there is no statistically-significant correlation between the value of K_t and cysticercoid age (r = -0.24, 36 degrees of freedom). In contrast, the fall in V_{max} value from 280 to 135 µmoles/g/h over the same age range is significantly correlated (r = -0.56 with 36 degrees of freedom; significant at p = 0.01). This would suggest that V_{max}, and hence the cycloleucine transporting capacity of the cysticercoid, declines as the parasite ages.

From the 3[illegible] kinetic experiments, a mean K_t value of 0.2[illegible] mM (range = 0.21-0.45 mM) and a mean V_{max} value of 220 µmoles/g/h (range = 160-351 µmoles/g/h) can be calculated. This mean data is presented as a Michaelis-Menten plot in Figure 4.5.2.5., and a Lineweaver-Burk plot in Figure 4.5.2.6.

Figure 4.5.2.1. Uptake of ^{14}C-cycloleucine by stage III cysticercoids. Each point is the mean of 12 replicates ± S.E.



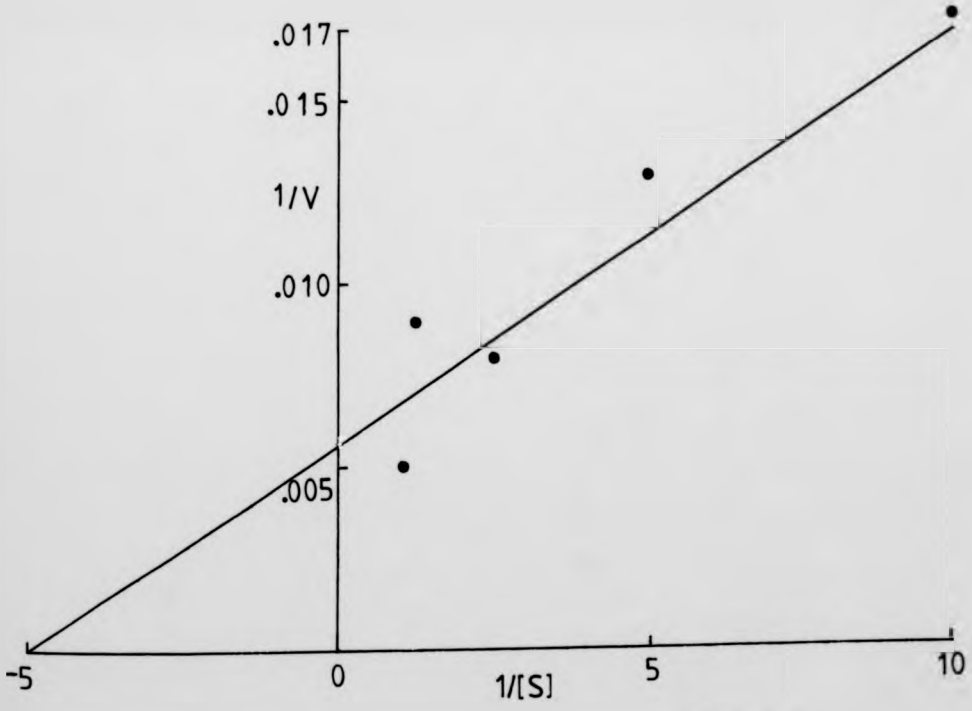


Figure 4.5.2.2. Lineweaver-Burk plot of Figure 4.5.2.1.

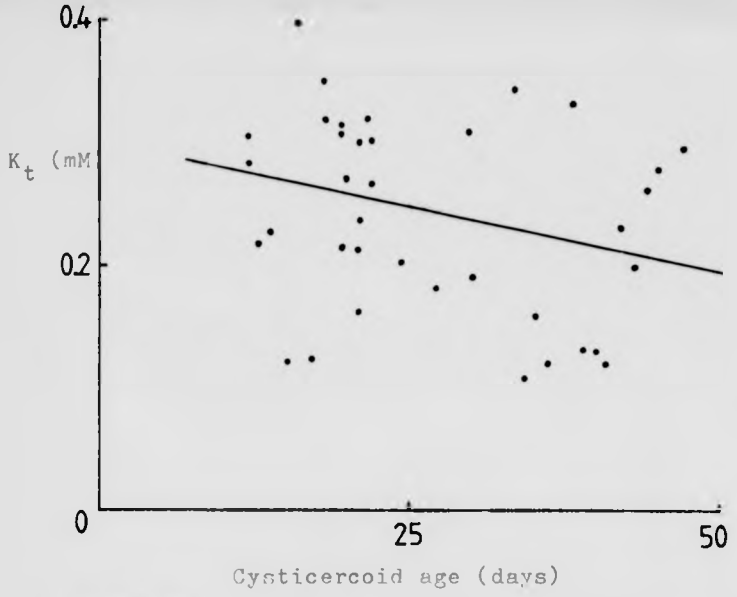


Figure 4.5.2.3. Scatter diagram of the relationship between cysticercoid age and K_t (for details, see text).

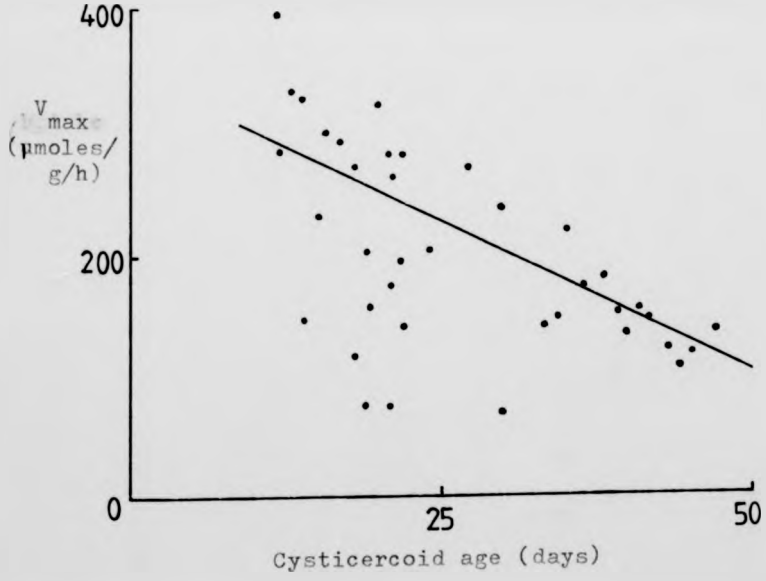


Figure 4.5.2.4. Scatter diagram of the relationship between cysticercoid age and V_{max} (for details, see text).

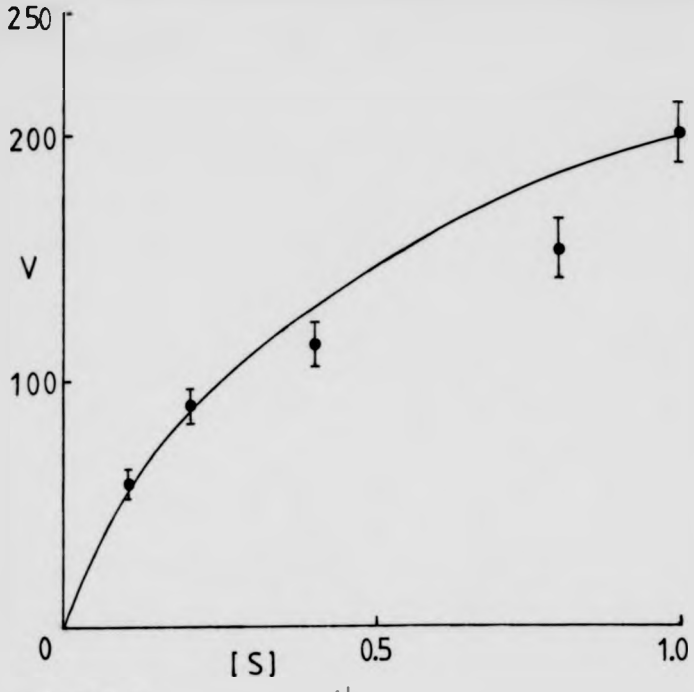


Figure 4.5.2.5. Uptake of ^{14}C-cycloleucine by stage V cysticercoids, aged 12-47. Each data point is the mean of 38 duplicate experiments, each of at least 5 replicates ± S.E.

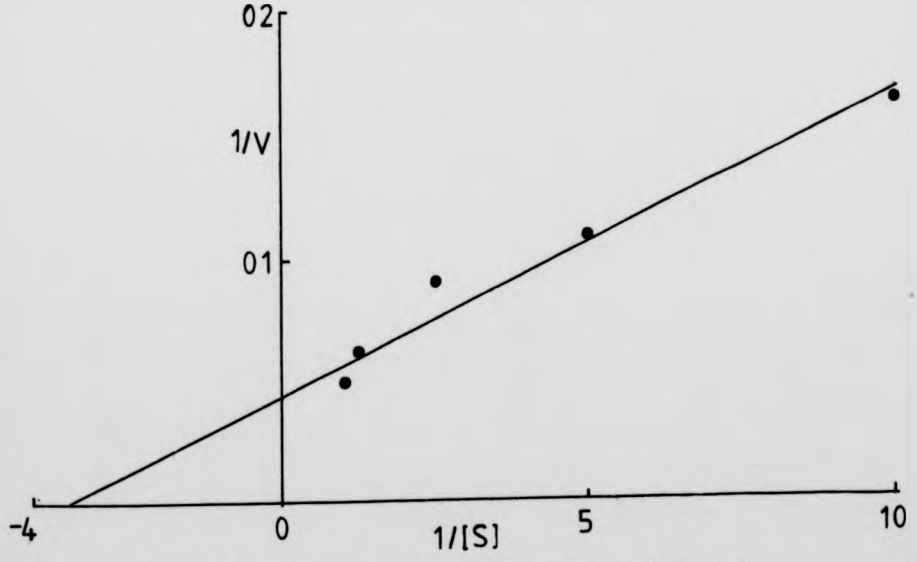


Figure 4.5.2.6. Lineweaver-Burk plot of Figure 4.5.2.6.

4.6. Discussion

The results presented in this section strongly indicate that cysticercoids absorb ^{14}C-cycloleucine by mediated transport mechanisms. Evidence for this is provided by the typical saturation isotherm obtained when the uptake of cycloleucine is examined at increasing concentrations, and the finding that α-AIB is an inhibitor of cycloleucine transport. Furthermore, the mechanism is one of active mediated transport (as opposed to facilitated diffusion, see Section 1) as cycloleucine is accumulated against a concentration gradient. There appears to be no diffusion component involved in the uptake of cycloleucine by stage III and stage V cysticercoids.

In many respects, the cycloleucine uptake locus(i) of the cysticercoid resembles that of the adult worm. Both actively absorb cycloleucine through carrier mediated systems, but the strongest evidence for similarity comes from an examination of the K_t and V_{max} values that describe the uptake of this compound. In both the adult worm, and both of the examined developmental stages of the cysticercoid, almost identical K_t values are noted (0.25 mM - adult; 0.28 mM, stage V cysticercoid; 0.21 mM, stage III cysticercoid). In addition, the range of V_{max} values obtained with the cysticercoids is comparable with the value of 170 µmoles/g/h obtained with the adult worm. A similar time-dependent absorption pattern of ^{14}C-cycloleucine is also apparent when the adult worm and stage V cysticercoid are compared, consisting of an almost linear uptake rate for 20

minutes, then a slow levelling-off to reach a steady state after about 45 minutes. Comparable concentration r tios after 10 minutes incubation in 0.1 mM ^{14}C-cycloleucine of c. 15, 9-17, and 18 were achieved by the adult worm, stage V and stage III cysticercoid respectively.

Figure 4.5.2.4. suggests that the pattern of ^{14}C-cycloleucine uptake changes as the cysticercoid ages. This may be a manifestation of the 'crowding effect', first mentioned in Section 2.4.7.1., where intraspecific competition for finite resources such as food or space may lead to alterations in patterns of metabolism which is manifested as a reduced rate of growth. A close correlation exists between the levels of certain nutrients, particularly glucose (Read, 1959), and the growth of tapeworms, and a number of studies have investigated the effects of parasite age, mass and infection density within the host on the ability of the adult H.diminuta to absorb nutrients. All of the following examples (except where indicated) concern this parasite. As a general rule, the rate of uptake of a nutrient declines as the mass of the worm increases, mass increase being generally correlated with worm aging. Examples are the uptake of methionine (Read et al., 1963), glucose (Henderson, 1977; Phifer, 1960a; Sterling, 1975; Roberts, 1980), glycerol (Pittman & Fisher, 1972) and galactose (Roberts, 1980). It is difficult to make direct comparisons between these experiments as worms of differing mass, age and derived from differing infection densities were used. All of these factors were found to have an effect on the uptake of the above-mentioned

compounds by the tapeworm. Some of these experiments involved kinetic analysis, and the results obtained are summarised in Table 4.6.1. In addition to the data of Table 4.6.1., Henderson (1977) found that the K_t value describing glucose uptake increased from 1 mM to 2 mM, and the V_{max} value fell from 101 to 48 μmoles/g/5 min, as worm dry weight increased from 5 to 60 mg. This is substantially in agreement with the results of Starling & Roberts (see Roberts, 1980). A further study by Read et al. (1963) found that the K_i values describing the interactions of various inhibitors of methionine uptake at the appropriate transport loci did not substantially alter as the worm aged. Henderson (1977) also found that the rate of glucose absorption fell with increasing infection density. This fall was in addition to the lowered uptake rate that could be attributed to the age and mass of the worms. Henderson did not attempt to measure the changes in the values of K_t and V_{max} that may have occured as the infection density was varied, but the results of this study have shown that K_t (cycloleucine) remains at the same value while the value of V_{max} (cycloleucine) falls as the number of cysticercoids within T.molitor rises (figures 2.4.7.6.1., 2.4.7.6.2.).

Thus the available evidence would suggest that as the mass of the worm increases, the value of V_{max} falls, and it is only in the cases of glucose and galactose absorption that the value of K_t rises, possibly to compensate. Such changes may well be related to changes in the metabolic needs of the parasite as it passes through the various stages of its life cycle. The growth

Table 4.6.1. Effects of age on the kinetic parameters describing the uptake of various substrates by H.diminuta

Substrate	Age (days)	K_t (mM)	V_{max}	Reference
Methionine	9	0.40	229[c]	1
	10	0.31	183	"
	11	0.64[b]	207	"
	12	0.39[b]	115	"
Glucose	6	0.43	49.8[d]	2
	10	0.74	20.1	"
	12	1.84	16.4	"
Galactose	6	1.89	62.3[d]	2
	10	2.70	25.2	"
	12	4.27	23.4	"
Cycloleucine[a]	12	0.27[b]	280[c]	3
	47	0.20[b]	135	"

a = Data for cysticercoids. All other data for adult worm.
b = No significant difference within data set. All other data sets are significantly different.
c = Units are µmoles/g/h
d = Units are µmoles/g/2 min
References: 1 = Read et al. (1963), 2 = Starling & Roberts (in Roberts, 1980), 3 = This study (see Section 4.5.2.).

of adult H.diminuta has been studied by Roberts (1961), who found that growth commences with a phase of exponential growth in length and mass over the first 1-7 days post-infection, followed by a period of retarded growth. Maximal size is reached after about 14 days. Thus, after a certain period of time, proportionately less nutrients may be needed for growth. This could be particularly pertinent to the cysticercoid of H.diminuta, as once it has reached the infective stage, it need no longer develop, and merely awaits ingestion by a suitable host. However, Figures 2.4.7.2.3. and 2.4.7.2.1. show that the cysticercoid continues to increase in both size and mass respectively for as long as it remains in the intermediate host. Ultrastructural studies have revealed that other changes are also taking place during this 'waiting' stage. The ultrastructure of the cysticercoid of H.diminuta after the scolex has been retracted has been extensively studied by Richards & Arme (1984). The findings of this study have been briefly summarised in Section 3.3.3., the major conclusions of which that are relevant to this section of the thesis being that the thickness of the tegument increases from 0.4 µm prior to scolex retraction to 1.1-1.4 µm after 9-10 days development at 26°C, to 3 µm after 13 days and finally as much as 16 µm after 46 days growth. Concurrent with this is an intrusion of fibrous bundles into the tegument which are very prominent after 25 days growth. However, microvilli are present throughout the ageing of the cysticercoid and the apical plasma membrane does not show changes.

The noted decline in the value of V_{max} is probably a reflection of the declining surface area/volume ratio, and can be

explained in the face of increasing growth by the following model. Consider a tapeworm of 10 mass units, bearing two transport loci, each of which mediates the uptake of 4 mass units of nutrients. If all of these nutrients are used for growth, and assuming no increase in the number and affinity of the loci, the pattern of growth will be a linear increase in mass (10 + 8 = 18, 18 + 8 = 26, 26 + 8 = 34 etc.), but as the rate of uptake is normally defined in terms of uptake per unit mass, it will show a decline as the mass of the worm increases (10/8 = 0.60, 18/8 = 0.38, 26/8 = 0.27 etc.). Although grossly simplified this model helps to explain how V_{max} could decline with growth. As V_{max} is normally taken to be a measure of the number of transporting loci per unit biomass, a declining V_{max} would reflect a decrease in the number of such loci. This may not actually happen, and to use a declining V_{max} value based on mass considerations as a measure of metabolic change is probably erroneous.

On the other hand, changes in K_t may reflect metabolic changes as the parasite ages. Starling & Roberts (in Roberts, 1980) found two systems mediating the transport of glucose by 6-day old adult H.diminuta, one of which was insensitive to K^+. This K^+ insensitive component was less apparent in 10-day old worms and could not be discerned in 20-day old worms. The noted increase in the value of K_t (glucose) as the worms aged is thus most likely to be a reflection of the changing proportions of kinetically distinct transport loci. These changes were found to occur along the entire length of the strobila of 6-, 10- and 20-day old worms, and thus the change is a reflection of whole

worm development, and is not confined to new proglottides produced by older worms. Another example of a K_t value, in this case describing the absorption of glucose by hamster small intestine, rising with increasing age is provided by the study of Honegger & Semenza (1971). These workers found that the change was due to the presence of two populations of glucose transport loci, one showing a low affinity for glucose and the other a high. As the hamster aged, the low affinity population increased at the expense of the high affinity population, thus resulting in an overall rise in the value of K_t. Adult H.diminuta has an absolute requirement for carbohydrate (see Section 7), but appears to fulfil its needs through a combination of behavioural and morphological factors. Presumably, less glucose is required once maturity is attained, so it is concievable that a lowering of the glucose transport loci affinities allows this to be achieved. Greater amounts of glucose are needed in the developing adult H.diminuta as it increases in weight considerably over 14 days (0.0005 mg-100 mg)(Roberts, 1961). As a result of the small surface area/volume ratio, and the presence of a large number of 'high-affinity' carbohydrate uptake loci, the 6-day old worm accumulates 56 mM glucose in its tissue fluids after 2 hours incubation in 2 mM glucose, compared to 28.5 mM for the 10-day old worm, and 20 mM for the 20-day old worm (Starling & Roberts, in Roberts, 1980).

In total contrast, the K_t values describing methionine and cycloleucine by the adult worm and cysticercoid respectively do not change with increasing age. This may reflect the need for

a constant amount of amino acids throughout the life of the parasite.

A change in the number of transport loci could explain the decline in uptake noted as the infection density of H.diminuta cysticercoids within Tenebrio increases (Figure 2.4.7.6.1.). Using H.diminuta as an example, nutrient transport loci are probably located on the brush border membrane of the tegument (see Section 1.) and the glycocalyx which overlies this membrane is replaced every 6-8 hours (Oaks & Lumsden, 1971). It is also known that the brush border membrane of S.mansoni is replaced every 24-42 hours (Kusel, Sher, Perez, Clegg & Smithers, 1975). If such a process also occurs in H.diminuta cysticercoids, there would have to be a continual synthesis of new transport loci to replace those lost when the membrane is shed. There is little evidence that tapeworms have the ability to synthesise either purines or pyrimidines de novo from simple precursors (Barrett, 1981), and, as a result, the worm has developed complex transport systems for the absorption of these compounds (MacInnis, Fisher & Read, 1965; MacInnis & Ridley, 1969; Pappas, Uglem & Read, 1973). If one further assumes that the transport loci are composed primarily of specific proteins, their synthesis must be dependent on the rate of RNA synthesis, which is ultimately dependent on the supply of RNA precursors. Thus a decline in the number of nucleotide transport systems would lead to a decline in the quantity of these compounds for RNA synthesis, a decline in the rate of protein synthesis, and thus an ultimate decline in the rate of growth due not only to a shortage of structural

proteins, but also of those proteins which form the nutrient transport systems. Bolla & Roberts (1971) have found that adult H.diminuta from high density infections have lower rates of nucleotide uptake, and a lower rate of protein synthesis. If such processes also occur in the cysticercoid, the V_{max} values describing the uptake of many compounds could fall, as a consequence of a reduced rate of protein synthesis leading to a smaller number of transport loci per unit biomass. However, unless the K_t values fell (i.e. increased affinity) to compensate, on the basis of this hypothesis, they should remain the same, whatever the infection density (c.f. Figure 2.4.7.6.2.).
It is also possible that the rate of lipid synthesis could be retarded by crowding, and as these compounds are essential components of biomembranes, including those of tapeworms (see Section 1.), growth may also be affected. As for nucleotides, adult H.diminuta has little ability to synthesis its own lipids and must absorb them ready-made from its ambient medium (Ginger & Fairbairn, 1966; Jacobsen & Fairbairn, 1967).

5. TRANSPORT OF AMINO ACIDS BY CYSTICERCOIDS - SITE (LOCUS) SPECIFICITY

5.1. Introduction

Inhibition studies allow conclusions to be drawn regarding the specificities of the various transport loci possessed by tapeworms. At the present time, adult H.diminuta has been shown to possess six separate loci for the transport of amino acids, with possibly a few more serving for the transport of neutral aromatic amino acids (see Introduction, Section 1.). In addition, the cysticercoid of H.diminuta has been shown to possess at least two amino acid transport loci, one for the transport of basic amino acids, and one for neutral amino acids. The relatively small inhibitory effect of aspartate upon α-AIB transport indicates a rather poor affinity of acidic amino acids for the neutral amino acid transport locus(i) (Arme & Coates, 1973).

This section presents the results of inhibition studies that have been designed initially to examine the specificity of the cycloleucine uptake loci, and then proceeds to examine the interactions between six amino acids (including cycloleucine) at the transport level from which conclusions may be drawn concerning the specificities of the various amino acid transport loci possessed by the cysticercoid. Experimental design is discussed in Section 5.2., the results given in Section 5.3., and discussed in Section 5.4.

5.2. Experimental design. Theoretical and practical considerations

5.2.1. General

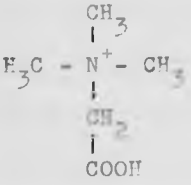
Inhibition analysis generally commences with an examination of the uptake of a single compound in the presence of a range of potential inhibitors. The I/S ratio (the ratio of inhibitor concentration to test compound concentration) is usually fixed, and although this form of analysis may show inhibition, the results do not enable the type of inhibition to be determined, and furthermore, may be biologically meaningless as the inhibitions shown at certain I/S ratios may never exist *in vivo*. A better system is to examine the uptake of the test compound over a range of I/S ratios (varying [S], keeping [I] fixed). This enables the type of inhibition to be determined and also allows the inhibition constant (K_i) to be calculated. Parity of K_t and K_i would suggest that both compounds share the same transport loci. Such an approach will provide information concerning the specificity of such loci that are available to the test compound only, but will not provide information concerning the number of loci that that compound actually uses. There are several approaches to solving this problem, but all involve studying the uptake of a group of structurally-similar compounds, and by examining their reciprocal interactions with each other, the number and specificities of the transport loci which serve to translocate this group can be determined.

In this section of the thesis, the compound chosen for the initial study is cycloleucine. As mentioned in the Introduction (Section 1.), it has the advantage of being metabolically inert (Section 2.4.6.), and in the adult H.diminuta enters the worm through the loci used by neutral amino acids. If the initial studies confirm that this is also the situation in the cysticercoid, the specificities of the loci serving for the uptake of cycloleucine can be examined in detail and compared to the equivalent loci possessed by the adult worm. The study will then proceed to determine the reciprocal interactions of cycloleucine with five other amino acids.

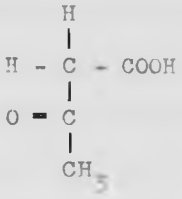
5.2.2. Initial study (Section 5.3.1.)

This section commences with an examination of the uptake of ^{14}C-cycloleucine by stage III and V cysticercoids in the presence of various potential inhibitors. For the amino acid inhibition studies, a relatively low I/S ratio has been employed (10 : 1 for stage V and 5 : 1 for stage III) as previous studies with the adult worm have shown 100% inhibitions of ^{14}C-cycloleucine uptake in the presence of certain amino acids at an I/S ratio of 20 : 1 (Harris & Read, 1968). However, for the non-amino acid/cycloleucine studies, a higher I/S ratio can be gainfully employed (250 : 1, in this instance) as there are few records of any interactions between amino acids and other compounds at even these high I/S ratios (Pappas & Read, 1975). As discussed in the Introduction (Section 1.), the structure of the

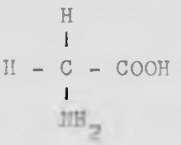
Figure 5.2.2.1. Structures of compounds used in Section 5.3.1. (For structures of cycloleucine, α-, β-, γ- and N-MeAIB, see Figure 1.9.)



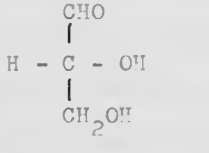
Betaine



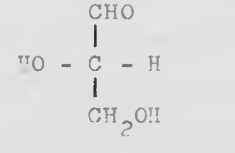
Acetylglycine



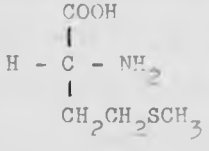
glycine



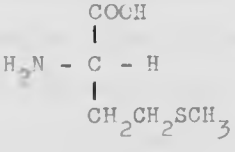
D-glyceraldehyde



L-glyceraldehyde



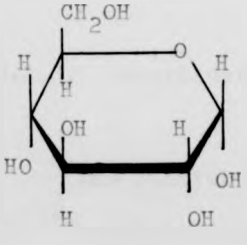
D-methionine



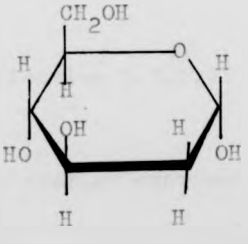
L-methionine

Stereoisomers of amino acids

By convention, the L- and D-isomers of amino acids are recognised by reference to the D- and L- isomers of glyceraldehyde, the smallest sugar with an asymmetric carbon atom.



D-glucose



2-Deoxy-D-glucose

(The structures of the other amino acids employed in this study, galactose, fructose, xylose and sodium acetate are not given).

inhibitor molecule determines its degree of interaction with a specific locus. In this study, the contributions that the various parts of the amino acid molecule make to this interaction are studied by observing the effects of amino acid analogues on the uptake of ^{14}C-cycloleucine. The contribution of the amino group is examined using the N-methyl analogue of α-AIB and the acetylated derivative of glycine to inhibit ^{14}C-cycloleucine uptake. The importance of the position of the amino group relative to the carboxyl group is examined by comparing the inhibitory effects of α-AIB and β-AIB upon ^{14}C-cycloleucine uptake, and the contributions of the side chain of the amino acid molecule by examining the effects of 18 different amino acids upon ^{14}C-cycloleucine uptake. These studies at a single I/S ratio should provide some information regarding the specificity of the cycloleucine uptake loci. For a more detailed study of the characteristics of these loci, the K_i values of several chosen amino acids acting as inhibitors of ^{14}C-cycloleucine uptake must be determined (Section 5.2.3.).

5.2.3. Determination of K_i values (Section 5.3.2.)

The initial experiment in this section examines the uptake of ^{14}C-cycloleucine at concentrations of 0.05, 0.1, 0.2, 0.4, and 0.8 mM in the presence of unlabelled 4 mM α-AIB. This provides I/S ratios of 80 : 1, 40 : 1, 20 : 1, 10 : 1 and 5 : 1. However, the results of this experiment (Figure 5.3.2.11.) showed the complete inhibition of cycloleucine uptake at I/S

ratios of 80 : 1, 40 : 1 and 20 : 1. As a result of this, all of the consequent experiments in this section examine the uptake of ^{14}C-cycloleucine at concentrations of 0.1, 0.2, 0.4, 0.8 and 1.0 mM in the presence of 2 mM unlabelled inhibitor, giving I/S ratios of 20 : 1, 10 : 1, 5 : 1, 2.5 : 1 and 2.0 : 1. For each experiment a Michaelis-Menten plot (v against [S]) will be drawn, and the values of K_t, K_i, and V_{max} calculated by the method of Lineweaver & Burk (see Sections 1. and 4.2.2.). The latter plot also allows the type of competitive interactions between cycloleucine and the inhibitor molecule to be determined. The amino acids chosen represent a wide range of structural diversity. All are L-isomers, except for D-methionine, which is included to determine whether stereospecificity exists in the cysticercoid loci. An examination of the uptake of ^{14}C-cycloleucine in the presence of unlabelled cycloleucine is a test for the presence of a diffusion component in the uptake of ^{14}C-cycloleucine. Non-parity of the K_t and K_i values would suggest that such a component exists, although this would argue against the findings of Figure 4.4.5., which suggests that the uptake of this compound occurs solely by mediated processes.

5.2.4. Time-dependent absorption of ^{14}C-amino acids other than cycloleucine (Section 5.3.3.)

Five amino acids were chosen for this part of the study for the following reasons:-

a.) α-AIB. Shares two of the same uptake loci as cycloleucine in the adult worm (Harris & Read, 1968).

b.) L-proline. Imino acid. Present in very high concentrations in Tenebrio haemolymph (Hurd, Browne & Arme, 1982).

c.) L-alanine. Aliphatic neutral amino acid. Enters through an 'A'-like locus in the adult worm (Read et al., 1963).

d.) L-leucine. Aliphatic neutral amino acid. Enters through an 'L'-like locus in the adult worm (Read et al., 1963).

e.) L-phenylalanine. Aromatic neutral amino acid. Enters through at least two'aromatic' loci in the adult worm (Pappas & Gamble, 1980).

In advance of kinetic studies with these compounds the initial rate period must be determined. Once established, kinetic studies can proceed. Accumulation can also be demonstrated.

5.2.5. Kinetic studies with amino acids other than cycloleucine

The same experimental method outlined in Section 5.2.3. is to be followed. In addition, the range of K_t and V_{max} values calculated by the method of Lineweaver & Burk can be found by reference to the method described in Section 4.2.2. In each case, K_i is calculated from the 'standard' values of

K_t and V_{max}.

5.2.6. Specificity of neutral amino acid transport loci (Section 5.3.5.)

First, the specificity of α-AIB for the cycloleucine transport loci, and the converse, will be determined. These two compounds have been shown to share the same loci in the adult worm (Harris & Read, 1968). The method employed is described in Section 4.2.3. In this instance, the uptake of 0.1 mM ^{14}C-α-AIB is examined in the presence of increasing concentrations of unlabelled cycloleucine. It has already been established that α-AIB completely inhibits the uptake of ^{14}C-cycloleucine (Figure 4.4.5.), and if unlabelled cycloleucine has the same effect on the uptake of ^{14}C-α-AIB, it may be assumed that both compounds enter through the same transport loci. In addition, this method will establish whether there is a diffusion component involved in the uptake of ^{14}C-α-AIB.

The major part of this section employs the methods of MacInnis et al. (1976) to examine the reciprocal inhibitory actions of the five amino acids mentioned in Section 5.2.4. plus cycloleucine. First, the uptake of the six ^{14}C-amino acids at a concentration of 0.2 mM in the presence of 2 mM unlabelled inhibitors (the same six amino acids) is determined, and an inhibition profile plotted. Next, the product moment correlation coefficients (r) describing the relationships

between all possible pairs of the amino acids are calculated. The statistical significance of these coefficients is fixed at the conventional level of $p = 0.05$, with three degrees of freedom. An examination of these coefficients allows the numbers and specificities of any transport loci to be described.

The final experimental section, 5.3.6., examines the uses of N-MeAIB and 2-ANC (see Introduction, Section 1.) for kinetic studies with tapeworms. This section, together with a discussion of the implications of its findings, is treated as a separate entity.

5.3. Results

5.3.1. Inhibition of ^{14}C-cycloleucine uptake by various compounds - initial study

Tables 5.3.1.1. and 5.3.1.4. show the percentage inhibition of ^{14}C-cycloleucine uptake in stage V and III cysticercoids respectively in the presence of a number of different amino acids. Table 5.3.1.2. shows the percentage inhibition of ^{14}C-cycloleucine uptake by stage V cysticercoids in the presence of compounds other than amino acids, and Table 5.3.1.3. shows the effects of various amino acid analogues upon ^{14}C-cycloleucine uptake. Tables 5.3.1.1. and 5.3.1.4. indicate that all of the amino acids tested, with the exceptions of L-lysine, L-glutamate and D-methionine, interact with the cycloleucine transport loci, and Table 5.3.1.2. indicates that such interactions are confined to amino acids. This would suggest that the loci which serve for the translocation of ^{14}C-cycloleucine are specific for amino acids, other compounds not being transported. Table 5.3.1.3. confirms that an intact amino group is essential if a compound is to be recognised by the cycloleucine transport loci, and that recognition is further dependent upon the relative position of this group with respect to the carboxyl group, the structure of the side chain (c.f. Table 5.3.1.1.) and which stereoisomer is being employed (c.f. results of D- and L-methionine upon ^{14}C-cycloleucine uptake). Betaine is also not recognised by these loci, and neither, apparently, is the free hydrogen atom at the α-carbon position

as both cycloleucine and α-AIB, which do not possess this structural feature, are absorbed or can inhibit the absorption of ^{14}C-cycloleucine respectively. The characteristics of the cycloleucine loci of the adult worm are given in Table 5.3.1.5. Table 5.3.1.6. reveals that there are considerable similarities between the loci possessed by stage III and V cysticercoids and the adult worm, the major difference being that glutamate does not interact with the cysticercoid cycloleucine uptake loci (at least at the single I/S ratio employed in this study) whereas this compound inhibits the uptake of ^{14}C-cycloleucine by 37% at an I/S ratio of 100 : 1 in the adult worm.

Table 5.3.1.1. Inhibition of 0.2mM ^{14}C-cycloleucine uptake by various amino acids (@ 2mM). Stage V cysticercoids.

Amino acid	Percentage inhibition
Cycloleucine	100.0
α-AIB	87.6
L-serine	82.4
L-glutamine	77.6
L-asparagine	72.6
L-threonine	61.1
Glycine	59.7
L-valine	51.1
L-alanine	51.1
L-proline	42.4
L-tryptophan	36.0
L-isoleucine	29.4
L-methionine	27.7
L-leucine	24.2
L-phenylalanine	22.2
L-cysteine	13.4
D-methionine	1.8*
L-lysine	0
L-glutamate	0

L-tyrosine was not tested due to solubility problems. Each result is the mean of at least five determinations, and all results are significantly different* to that obtained with ^{14}C-cycloleucine alone at p=0.05. All incubations for 5 minutes.

* denotes D-methionine result is NOT significantly different from result obtained in the absence of inhibitor.

Table 5.3.1.2. Inhibition of ^{14}C-cycloleucine uptake (0.4 mM) by various potential inhibitors (@ 100 mM). Stage V cysticercoids

Inhibitor	Percentage Inhibition
D-glucose	0
D(+)-galactose	0
β(D)-fructose	0
D(+)-xylose	0
2-deoxy-D-glucose	0
sodium acetate	0

Each result is the mean of at least 8 determinations. All results do not differ significantly (@ $p = 0.05$) from the result obtained with 0.4 mM ^{14}C-cycloleucine alone. All incubations for 5 minutes.

Table 5.3.1.3. Effects of various amino acid analogues upon the uptake of ^{14}C-cycloleucine

Addition[+]	n	[S] (mM)	[I] (mM)	% Inhibition
α-AIB	10	0.1	2	71.1
β-AIB	9	"	"	1.1*
MeAIB	10	"	"	1.0*
glycine	9	"	"	40.2
acetyl-glycine	9	"	"	0
betaine	7	0.4	100	0

[+]see section 5.2.2.1. for structures.

*denotes result NOT significantly different ($p = 0.05$) from that obtained in absence of inhibitor.

Table 5.3.1.4. Inhibition of 1.0mM ^{14}C-cycloleucine uptake by various amino acids (@5mM). Stage III cysticercoids.

Inhibitor	Percentage inhibition
Cycloleucine	93.4
L-proline	49.2
L-alanine	46.4
L-leucine	44.4
L-phenylalanine	38.8
L-methionine	34.5
L-glutamic acid	0

Each result is the mean of at least 8 determinations. All incubations for five minutes. All results are significantly different from those obtained with ^{14}C-cycloleucine alone at $p = 0.05$.

Table 5.3.1.5. Inhibitions of 0.1mM ^{14}C-cycloleucine uptake by various amino acids (@ 2mM; except L-glutamate @ 10mM). Data is for adult H.diminuta, taken from HARRIS & READ (1968).

Amino acid	Percentage inhibition
α-AIB	61.4
L-alanine	55.3
L-proline	46.8
L-valine	46.3
L-leucine	38.3
L-phenylalanine	15.4
L-glutamate	36.6
L-lysine	0

Each result is the mean of 4-12 determinations.

Table 5.3.1.6. Order of effectiveness of various amino acids as inhibitors of ^{14}C-cycloleucine uptake (highest first).

Adult worm[+]	Stage V cysticercoid	Stage III cysticercoid
α-AIB	α-AIB	cyclo
ala	val	pro
pro	ala	ala
val	pro	leu
leu	leu	phe
phe	phe	met
glu	glu & lys	glu
lys		

[+] data from HARRIS & READ (1968).

5.3.2. Determination of K_i values

A summary of the experiments performed (all with stage V cysticercoids), and the results obtained, is given in Table 5.3.2.1. The percentage inhibition of ^{14}C-cycloleucine uptake in the presence of each inhibitor at the various I/S ratios is given in Table 5.3.2.2. These values have been calculated from the lines of best fit from the appropriate Lineweaver-Burk plots. The values in brackets are extrapolated from these plots. The relative activities of each of the 11 amino acids tested as inhibitors of ^{14}C-cycloleucine uptake are best examined as reciprocals of K_i, and an inhibition profile is presented in Figure 5.3.2.11.

The results indicate that all of the amino acids tested, with the exceptions of L-lysine, L-glutamate and D-methionine, are competitive inhibitors of ^{14}C-cycloleucine uptake. Figure 5.3.2.11. shows that the greatest inhibition is caused by α-AIB, followed by unlabelled cycloleucine, then L-alanine, glycine, L-proline, L-lysine, L-leucine, L-methionine and L-phenylalanine. The order of these results are similar to those obtained at a single I/S ratio (Table 5.3.1.1.). Although the K_i value calculated for the effects of L-lysine on ^{14}C-cycloleucine uptake suggests that this compound has some affinity for the cycloleucine uptake loci, the form of the Michaelis-Menten plot (Figure 5.3.2.10.1.) would suggest that this compound has no significant inhibitory effects on the uptake of ^{14}C-cycloleucine. Figure 5.3.2.1.3. indicates that unlabelled cycloleucine has a strong

inhibitory effect on the uptake of ^{14}C-cycloleucine, and the Lineweaver-Burk plot of this data (Figure 5.3.2.1.4.) shows that this inhibition is competitive in nature. The calculated K_i value of 0.21 mM compares closely to the reference K_t value of 0.28 mM, and this evidence, coupled with the findings of Figure 4.4.5., strongly suggests that there is no diffusion component involved in the uptake of cycloleucine by the stage V cysticercoid of *H.diminuta*.

Table 5.3.2.1. Details of K_i experiments (see text for further details). The value of K_t is taken from the 'standard' value obtained from stage V cysticercoids aged between 12-47 days (figure 4.5.2.6.).

Inhibitor	n	K_t (mM)	K_i (mM)	Nature of inhibition	Figure nos.	
					M/M plot	L/B plot
cyclo (4 mM)	10	0.28	-+	-	5.3.2.1.1.	5.3.2.1.2.
cyclo (2 mM)	10	"	0.21	competitive	5.3.2.1.3.	5.3.2.1.4.
α-AIB	5	"	0.19	competitive	5.3.2.2.1.	5.3.2.2.2.
L-ala	15	"	0.64	competitive	5.3.2.3.1.	5.3.2.3.2.
gly	15	"	0.85	competitive	5.3.2.4.1.	5.3.2.4.2.
L-pro	15	"	2.08	competitive	5.3.2.5.1.	5.3.2.5.2.
L-leu	25	"	3.21	competitive	5.3.2.6.1.	5.3.2.6.2.
L-met	25	"	7.52	competitive	5.3.2.7.1.	5.3.2.7.2.
D-met	25	"	14.6	none	"	"
L-phe	20	"	3.66	competitive	5.3.2.8.1.	5.3.2.8.2.
L-glu	15	"		none	5.3.2.9.1.	5.3.2.9.2.
L-lys	5	"	2.70	none*	5.3.2.10.1.	5.3.2.10.2.

+ denotes insufficient data points to calculate K_i

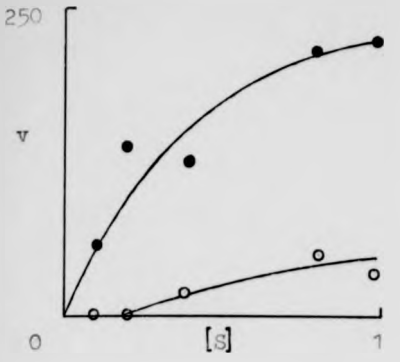
* see text regarding L-lysine result

Table 5.3.2.2. Percentage Inhibition of ^{14}C-cycloleucine uptake by different inhibitors at increasing I/S ratios.

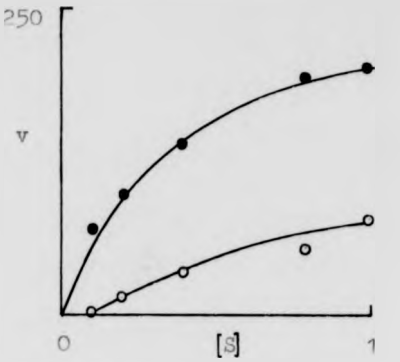
Inhibitor	I/S ratio					
	20 : 1	10 : 1	5 : 1	2.5 : 1	2.0 : 1	(1 : 1)
Cycloleucine	100	100	87.1	85.2	84.7	(82.5)
α-AIB	100	87.6	84.7	80.5	78.9	(74.0)
L-alanine	63.2	51.1	28.2	0	0	(0)
Glycine	64.2	59.7	53.8	47.3	45.4	(27.0)
L-proline	45.1	42.4	39.3	35.9	35.2	(32.9)
L-leucine	29.6	24.2	17.4	11.1	9.3	(5.0)
L-methionine	29.2	27.7	22.6	16.9	15.2	(10.1)
D-methionine	6.1*	1.8*	0	0	0	(0)
L-phenylalanine	34.4	26.2	19.4	14.0	12.7	(4.5)
L-glutamic acid	0	0	0	0	0	(0)
L-lysine	21.3*	13.7*	5.4*	0	0	(0)

* denotes result not significant at p=0.05 from uptake in absence of L-lysine

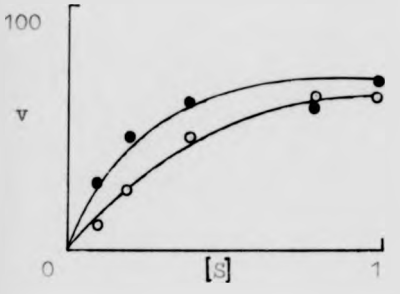
Data calculated from lines of best fit from the appropriate Lineweaver-Burk plots. Figures in brackets are extrapolated from these plots.



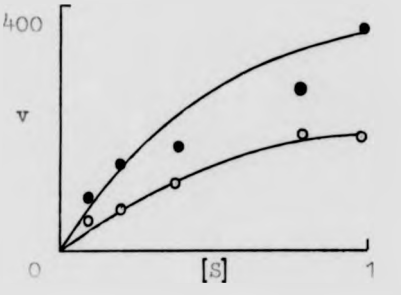
5.3.2.1.3. I = 2 mM cyclo



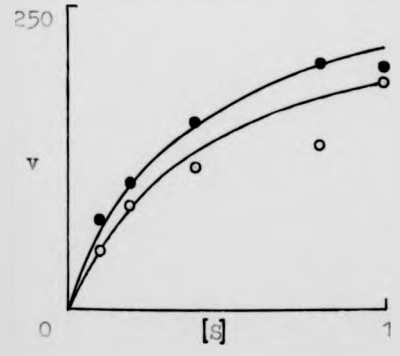
5.3.2.2.1. I = 2 mM α-AIB



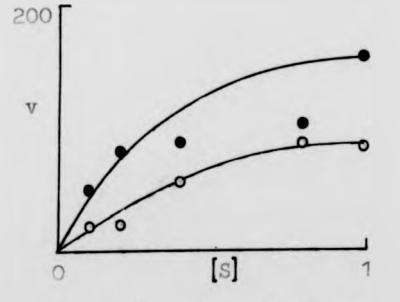
5.3.2.3.1. I = 2 mM L-ala



5.3.2.5.1. I = 2 mM L-pro

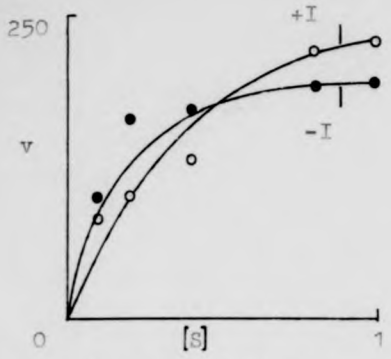


5.3.2.6.1. I = L-leu (2 mM)

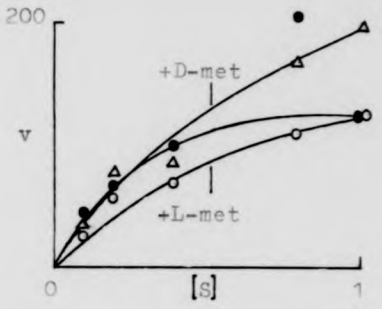


5.3.2.4.1. I = 2 mM gly

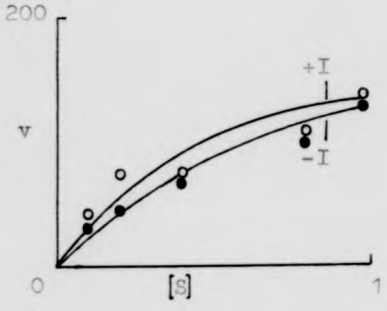
Figures 5.3.2.1.1.-5.3.2.10.1. Inhibition of ^{14}C-cycloleucine uptake by various inhibitors (I). In all plots; -●- = ^{14}C-cyclo alone, -o- = ^{14}C-cyclo plus inhibitor (plots continue on next page).



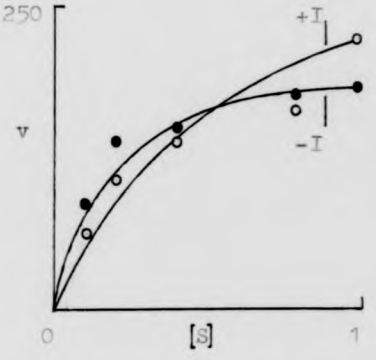
5.3.2.8.1. I = 2 mM L-phe



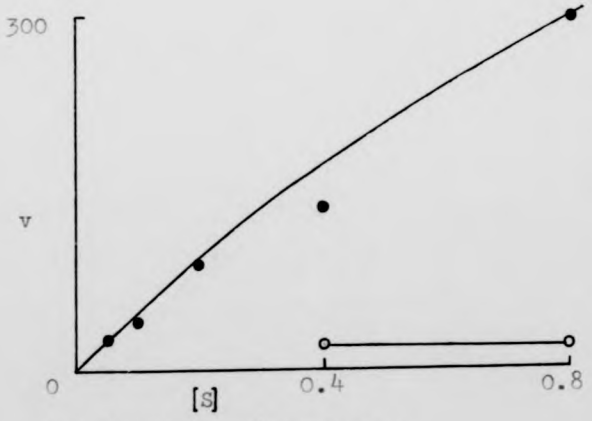
5.3.2.7.1. I = 2 mM L-met (-o-) or D-met (-Δ-)



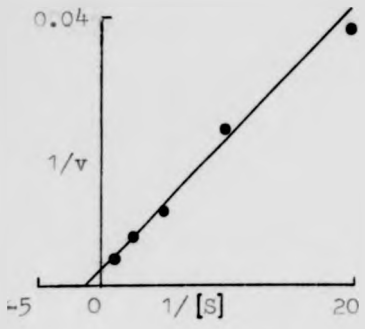
5.3.2.9.1. I = 2 mM L-glu



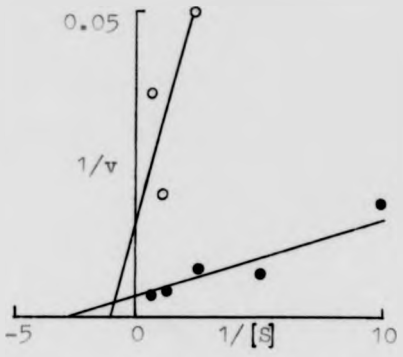
5.3.2.10.1. I = 2 mM L-lys



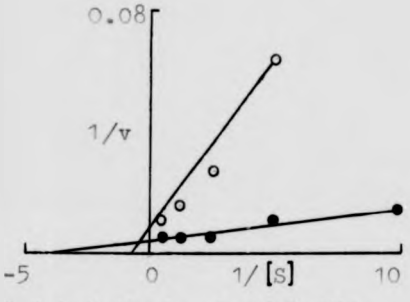
5.3.2.1.1. I = 4 mM cyclo



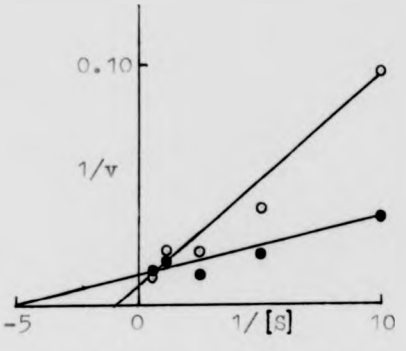
5.3.2.1.2. I = 4 mM cyclo



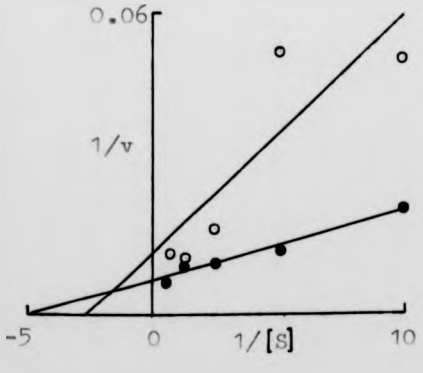
5.3.2.1.4. I = 2 mM cyclo



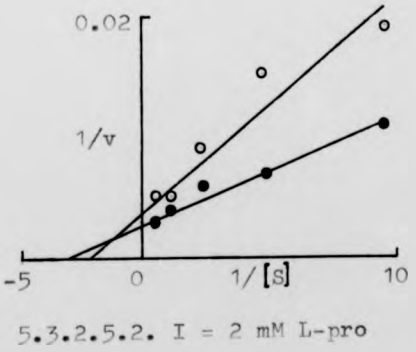
5.3.2.2.2. I = 2 mM α-AIB



5.3.2.3.2. I = 2 mM L-ala

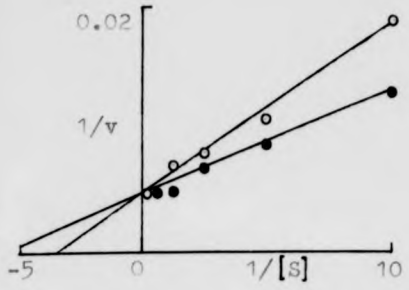


5.3.2.4.2. I = 2 mM gly

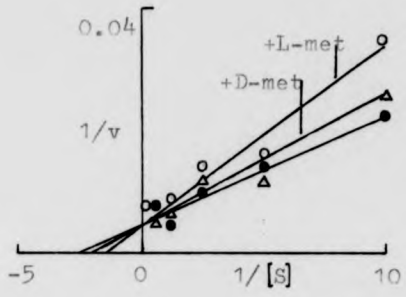


5.3.2.5.2. I = 2 mM L-pro

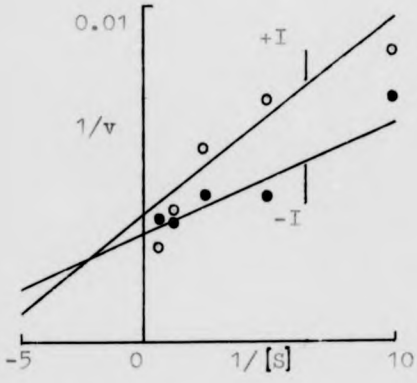
Figures 5.3.2.1.2.-5.3.2.10.2. Lineweaver-Burk plots of data in Figures 5.3.2.1.1.-5.3.2.10.1. -●- = ^{14}C-cyclo alone, -o- = ^{14}C-cyclo plus inhibitor (plots continue on next page).



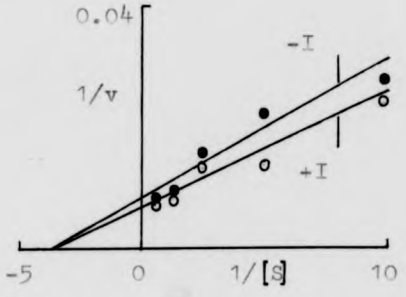
5.3.2.6.2. I = 2 mM L-leu



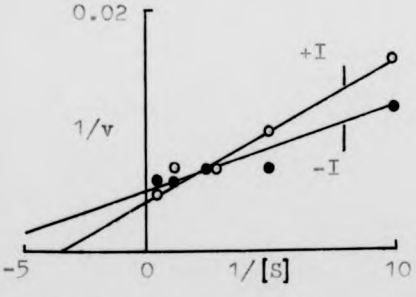
5.3.2.7.2. I = 2 mM L-met (-o-) or D-met (-Δ-)



5.3.2.8.2. I = 2 mM L-phe



5.3.2.9.2. I = 2 mM L-glu



5.3.2.10.1. I = 2 mM L-lys

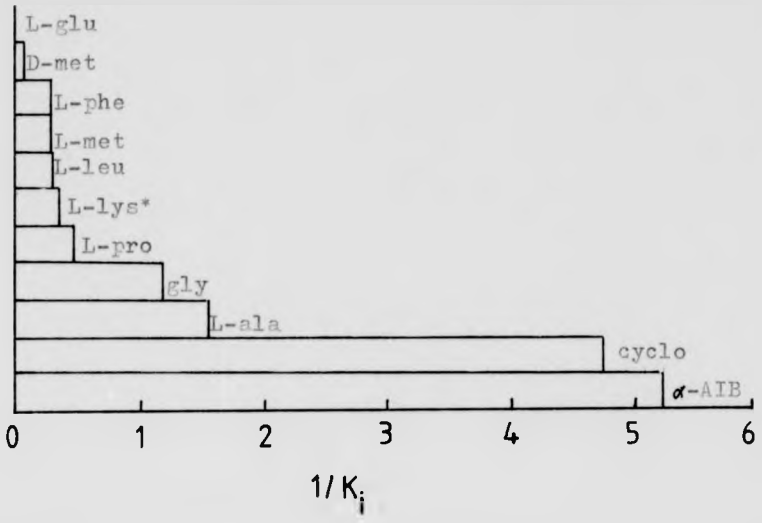


Figure 5.3.2.11. Relative activity of amino acids as inhibitors of ^{14}C-cycloleucine uptake (* see text regarding L-lysine result).

5.3.3. Time-dependent absorption of ^{14}C-amino acids other than cycloleucine by stage V cysticercoids

Five ^{14}C-labelled amino acids (α-AIB, L-proline, L-alanine, L-leucine and L-phenylalanine) were employed in this study. All of them have been previously shown to inhibit competitively the uptake of cycloleucine (Section 5.3.2.). The time-dependent absorption of these compounds, as well as cycloleucine, at a concentration of 0.2 mM, is presented in Figure 5.3.3.1. The apparent concentration ratios achieved at each data point (possible metabolism has not been determined, except for cycloleucine (Section 2.4.6.) and leucine (Section 6.3.1.)) are listed in Table 5.3.3.1.

Figure 5.3.3.1. indicates that the uptake of all the compounds except L-phenylalanine and L-leucine is approximately linear with respect to time for 10 minutes, and shows little deviation from linearity over 30 minutes. The reason for the apparent peaks in phenylalanine and leucine uptake after 1 and 5 minutes respectively is unknown although they appear regularly in repeated experiments. As the absorption of all the compounds appeared to be linear with respect to time for at least 5 minutes, this time period was adopted for the kinetic studies that comprise Section 5.4. From Table 5.3.3.1. it is apparent that all of the compounds are accumulated within the cysticercoid.

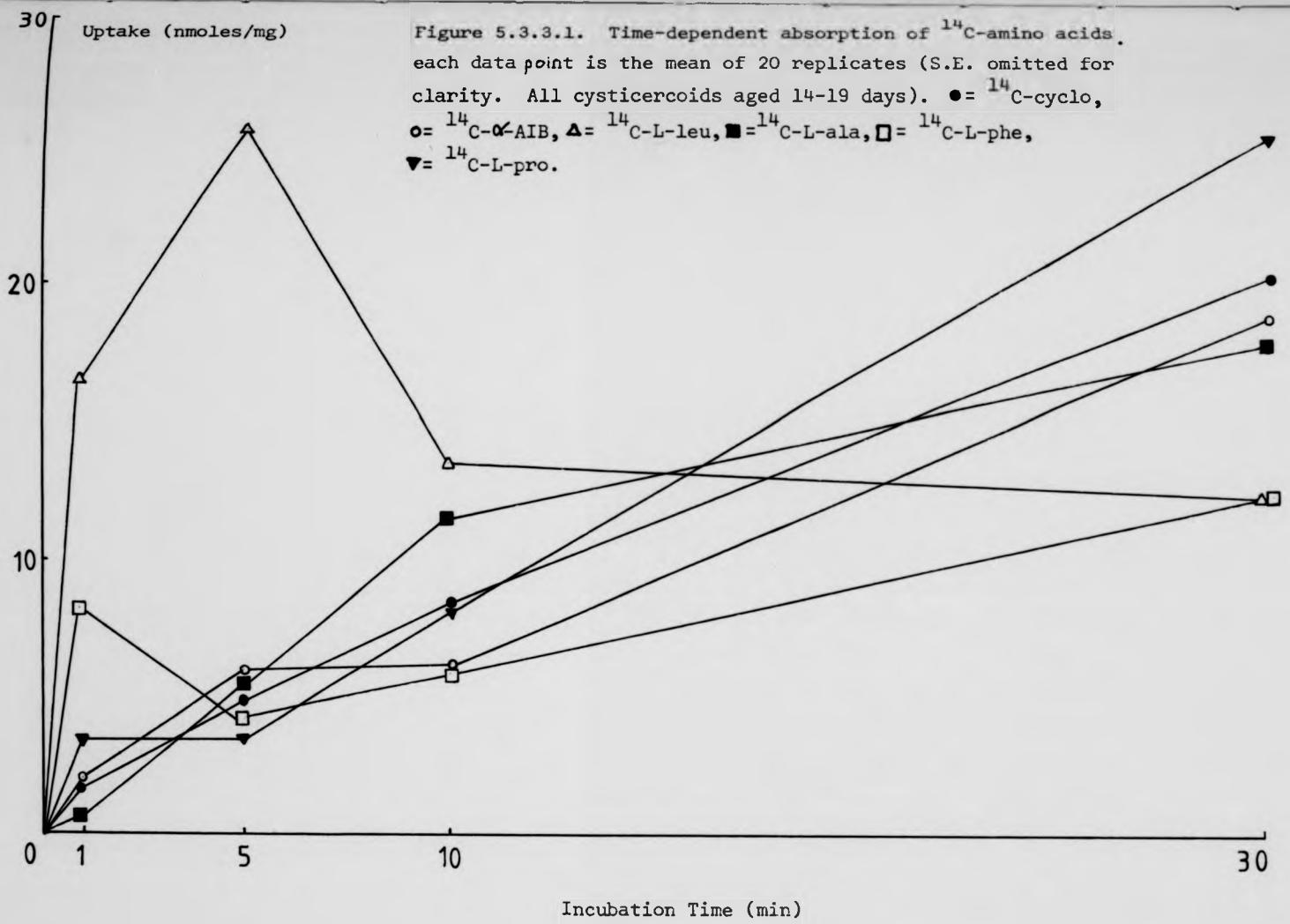


Figure 5.3.3.1. Time-dependent absorption of ^{14}C-amino acids. each data point is the mean of 20 replicates (S.E. omitted for clarity. All cysticercoids aged 14-19 days). ●= ^{14}C-cyclo, ○= ^{14}C-α-AIB, △= ^{14}C-L-leu, ■= ^{14}C-L-ala, □= ^{14}C-L-phe, ▼= ^{14}C-L-pro.

Table 5.3.3.1. Concentration ratios obtained with various ^{14}C-amino acids by stage V cysticercoids.

Amino acid	Time (minutes)			
	1	5	10	30
Cycloleucine	2.34	6.12	10.56	25.26
α-AIB	2.58	7.54	7.95	23.52
L-leucine	19.18	31.95	16.93	15.57
L-alanine	0.99	6.82	14.56	24.97
L-phenylalanine	10.21	5.39	7.46	14.20
L-proline	4.23	4.39	10.27	31.58

5.3.4. Kinetic studies with ^{14}C-amino acids other than cycloleucine

The uptake of 0.1 - 1.0 mM α-AIB, L-alanine, L-proline, L-phenylalanine, and L-leucine are presented as Michaelis-Menten plots in Figures 5.3.4.1.1.-5.3.4.1.5., and Lineweaver-Burk plots in Figures 5.3.4.2.1.-5.3.4.2.5. The uptake of 0.05 - 0.8 mM α-AIB is similarly presented in Figures 5.3.4.1.6. and 5.3.4.2.6. The values of K_t and V_{max} obtained from the Lineweaver-Burk plots are given in Table 5.3.4.1.

The inhibitory effects of unlabelled cycloleucine on the uptake of the five amino acids mentioned above was examined by the method of Lineweaver & Burk, and the appropriate K_i values calculated. The 'substrate' concentration range was 0.1 - 1.0 mM for all five amino acids, plus one additional experiment employing α-AIB at concentrations of between 0.05 - 0.8 mM. The concentration of unlabelled cycloleucine was fixed at 2 mM, giving a range of I/S ratios between 20 : 1 and 2 : 1, although in the experiment employing 0.05 - 0.8 mMα-AIB, 4 mM unlabelled cycloleucine was used, giving an I/S range of 80 : 1 to 5 : 1. Michaelis-Menten plots of these experiments are given as Figures 5.3.4.3.1.-5.3.4.3.5. ([S] = 0.1 - 1.0 mM) and 5.3.4.3.6. (α-AIB = 0.05 - 0.8 mM), and the Lineweaver-Burk plots as Figures 5.3.4.4.1.-5.3.4.4.5. ([S] = 0.1 - 1.0 mM) and 5.3.4.4.6. (α-AIB = 0.05 - 0.8 mM). A summary of the mode of inhibition, K_i values, $1/K_i$ values and a comparison with the appropriate K_t values is given in Table 5.3.4.2. Table 5.3.4.3. shows the percentage inhibition of the five amino acids uptake in the

presence of 2 mM cycloleucine as the I/S ratio increases, and Table 5.3.4.4. compares these percentage inhibitions with the pertinent results obtained with the adult worm. A comparison of the K_t and V_{max} values obtained with the six amino acids employed in this study between the adult and cysticercoid stages of M.diminuta is given in Table 5.3.4.5.

The results indicate that all five amino acids are absorbed by mediated processes, uptake being non-linear with respect to concentration. With the exception of the result obtained with ^{14}C-L-proline, the K_t and V_{max} values obtained with the cysticercoid are comparable to the equivalent values obtained with the adult worm. Inhibition studies reveal that cycloleucine interacts competitively with the loci serving for the transport of α-AIB, L-alanine, L-proline and L-leucine, but not with those transporting L-phenylalanine. A similar situation exists in the adult worm, except that in this case cycloleucine does interact with the phenylalanine-transporting loci. A further investigation of the interactions between ^{14}C-L-phenylalanine and cycloleucine was performed, but even at I/S ratios of 40 : 1 and 80 : 1 the uptake of phenylalanine was unimpaired.

When the results of this section are compared with those of Section 5.3.2., and the relative activities of either cycloleucine or the other five amino acids as inhibitors of the uptake of either the other five amino acids or cycloleucine respectively examined, one finds in the former case an order of inhibitory activity of (strongest first) of α-AIB>L-ala>L-leu>

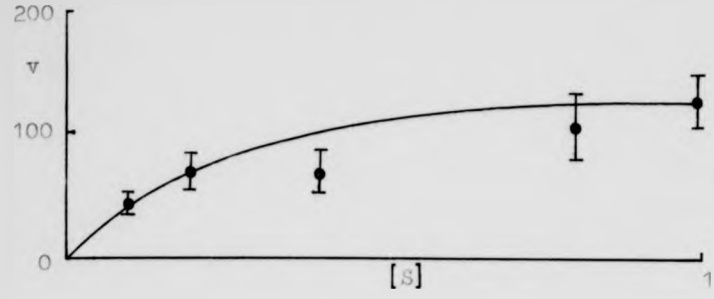
Table 5.3.4.1. Results of kinetic experiments

	^{14}C-amino acid		
	α-AIB	α-AIB	L-ala
n	20	20	10
[S] [b]	0.05-0.8	0.1-1.0	0.1-1.0
K_t (standard) [c]	0.47	0.35	0.30
K_t (range)	0.36-0.68	0.22-0.93	0.16-4.03
V_{max} (standard)	160	285	184
V_{max} (range) [d]	122-232	176-752	96-2500
M/M plot	5.3.4.1.1.	5.3.4.1.6.	5.3.4.1.2.
L/B plot	5.3.4.2.2.	5.3.4.2.6.	5.3.4.2.2.
	L-pro	L-phe	L-leu
n	15	15	25
[S]	0.1-1.0	0.1-1.0	0.1-1.0
K_t (standard)	0.88	0.20	0.32
K_t (range)	(a)	0.17-0.20	0.27-0.33
V_{max} (standard)	362	132	298
V_{max} (range)	(a)	115-155	256-304
M/M plot	5.3.4.1.3.	5.3.4.1.4.	5.3.4.1.5.
L/B plot	5.3.4.2.3.	5.3.4.2.4.	5.3.4.2.5.

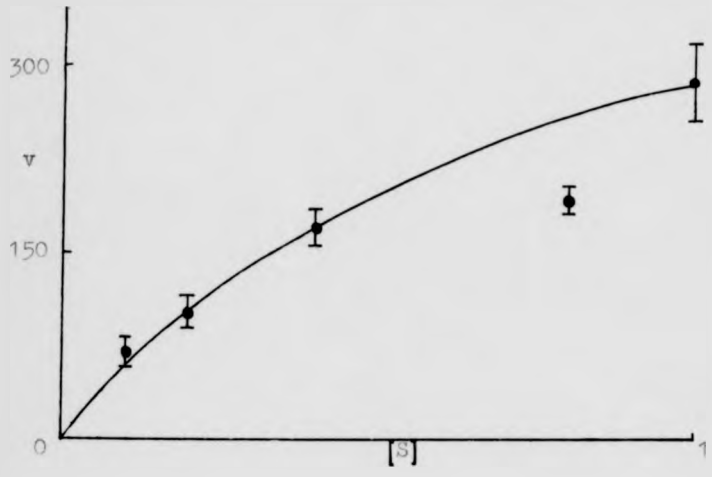
a = No error in straight line plot (r = 1.000)

b, c = units of concentration range [S] and K_t = mM

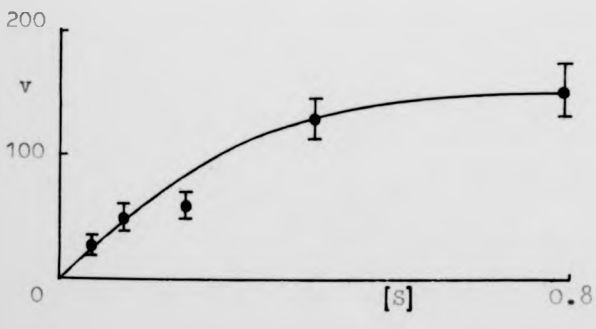
d = units of V_{max} = μmoles/g/h



5.3.4.1.4.
L-phenylalanine

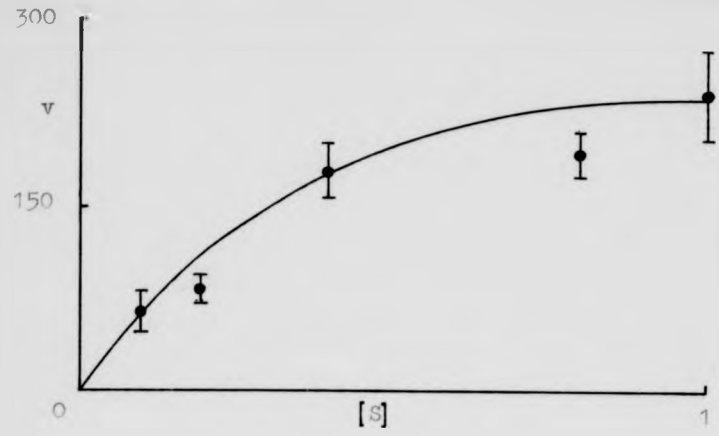


5.3.4.1.5.
L-leucine



5.3.4.1.6.
α-AIB

Figures 5.3.4.1.4.-5.3.4.1.6. Uptake of ^{14}C-L-phenylalanine, leucine and α-AIB by cysticercoids of H.diminuta. Michaelis-Menten plots.



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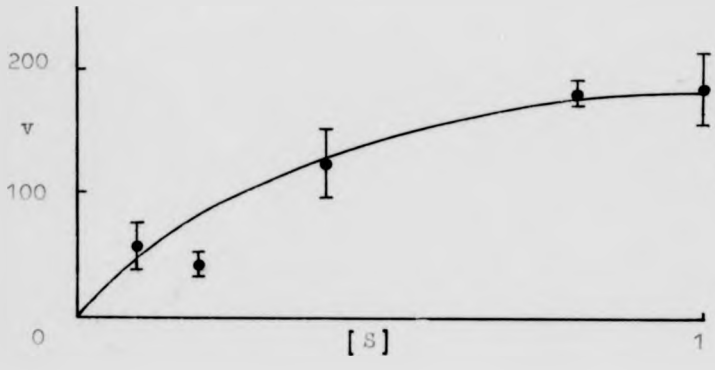
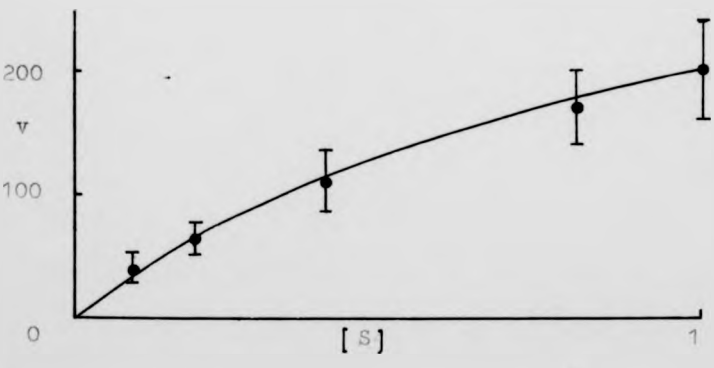
α-AIB



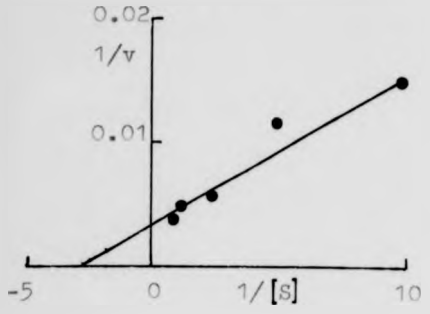
5.3.4.1.2.
L-alanine



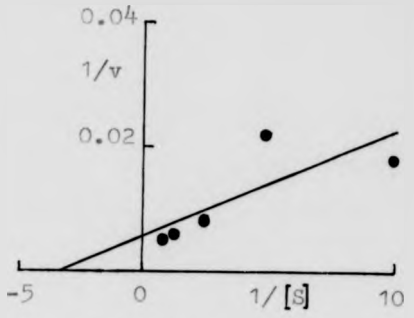
5.3.4.1.3.
L-proline

Figures 5.3.4.1.1.-5.3.4.1.3. Uptake of ^{14}C-L-alanine, proline and α-AIB by cysticercoids of H.diminuta. Michaelis-Menten plots.

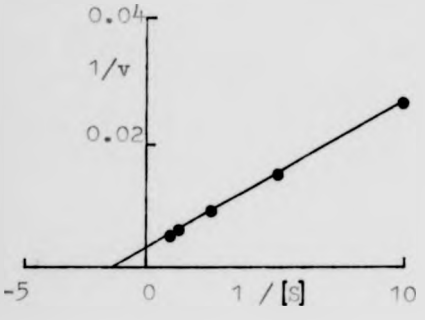
Figures 5.3.4.2.1.-6. Lineweaver-Burk plots of Figures 5.3.4.1.1.-6.



5.3.4.2.1. α-AIB



5.3.4.2.2. L-alanine



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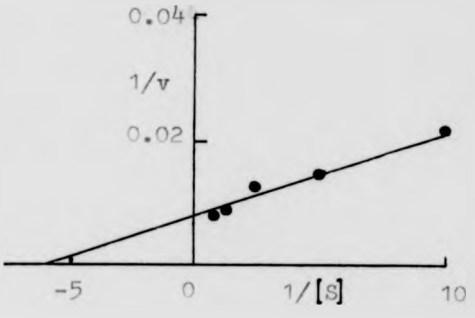
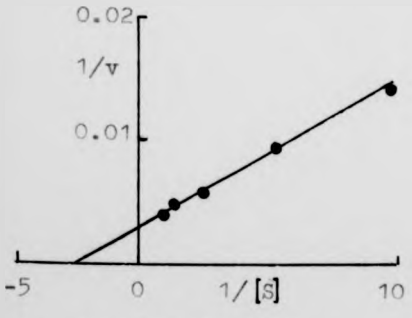
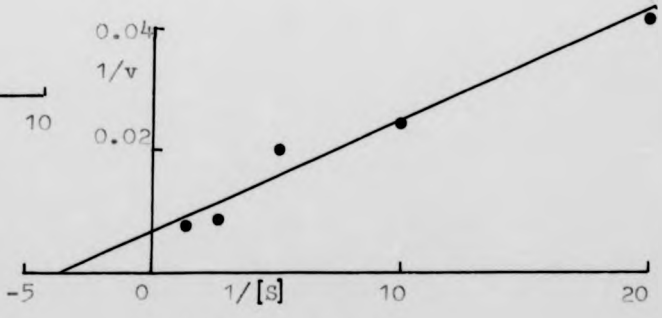


5.3.4.2.4. L-phenylalanine



5.3.4.2.5. L-leucine



5.3.4.2.6. α-AIB

Table 5.3.4.2. Inhibition of ^{14}C-amino acid uptake by 2 mM (*4 mM) unlabelled cycloleucine

^{14}C-amino acid	K_t(mM)	K_i(mM)	$1/K_i$	nature of inhibition	Figure nos.	
					M/M plot	L/B plot
α-AIB *	0.47	(a)	(a)	(a)	5.3.4.3.6.	(a)
α-AIB	0.35	0.11	9.09	comp	5.3.4.3.1.	5.3.4.4.1.
L-ala	0.30	0.15	6.67	comp	5.3.4.3.2.	5.3.4.4.2.
L-pro	0.88	1.50	0.67	comp	5.3.4.3.3.	5.3.4.4.3.
L-phe	0.20	(b)	(b)	(b)	5.3.4.3.4.	5.3.4.4.4.
L-leu	0.32	0.83	1.21	comp	5.3.4.3.5.	5.3.4.4.5.

a = K_i value not calculated (too few data points)

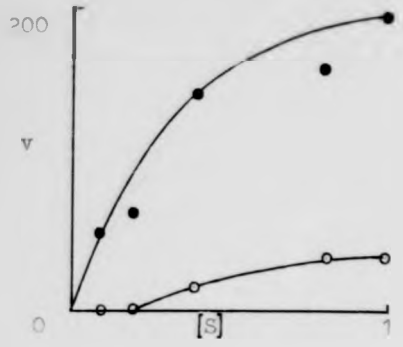
b = cycloleucine does not inhibit ^{14}C-L-phenylalanine uptake

comp = competitive inhibition

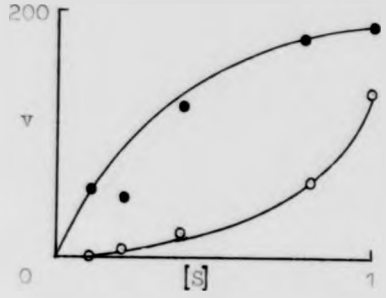
Table 5.3.4.3. Percentage inhibition of various ^{14}C-amino acids uptake by 2mM cycloleucine at increasing I/S ratios

^{14}C-amino acid	I/S ratio					
	20 : 1	10 : 1	5 : 1	2.5 : 1	2.0 : 1	(1 : 1)
α-AIB	(93.5)	(91.8)	86.4	80.5	76.0	(43.9)
L-alanine	(90.1)	86.8	78.7	52.3	28.5	(0)
L-proline	(56.6)	(56.1)	55.4	54.4	54.1	(53.1)
L-leucine	(68.6)	67.3	65.6	63.9	63.5	(62.2)
L-phenylalanine	0	0	0	0	0	0

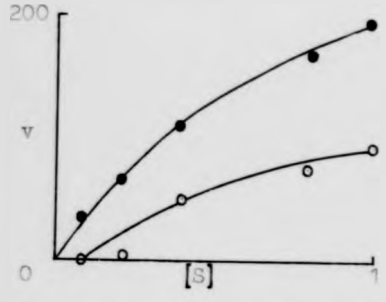
Data calculated from lines of best fit from the appropriate Lineweaver-Burk plots. Figures in brackets are extrapolated from these plots.



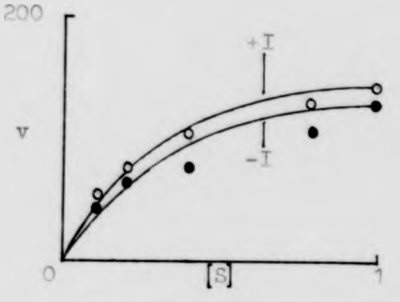
5.3.4.3.1. α-AIB + 2 mM I



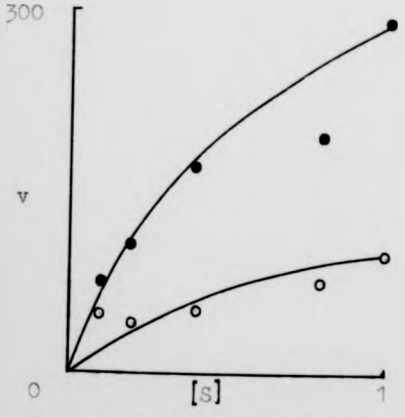
5.3.4.3.2. L-ala + 2 mM I



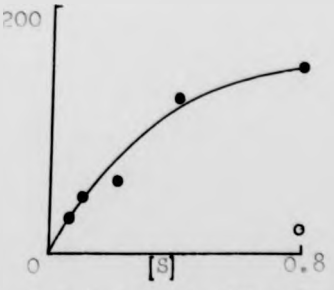
5.3.4.3.3. L-pro + 2 mM I



5.3.4.3.4. L-phe + 2 mM I

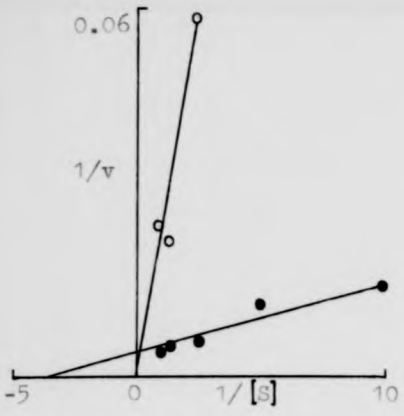


5.3.4.3.5. L-leu + 2 mM I

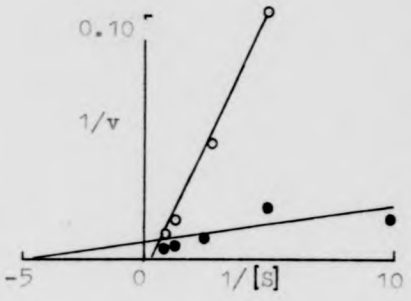


5.3.4.3.6. α-AIB + 4 mM I

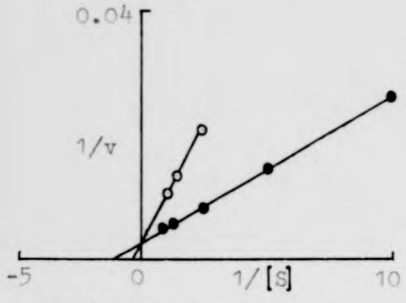
Figures 5.3.4.3.1.-5.3.4.3.6. Inhibition of uptake of various ^{14}C-amino acids by unlabelled cycloleucine (I). -●- = ^{14}C-amino acid alone; -o- = ^{14}C-amino acid plus cycloleucine.



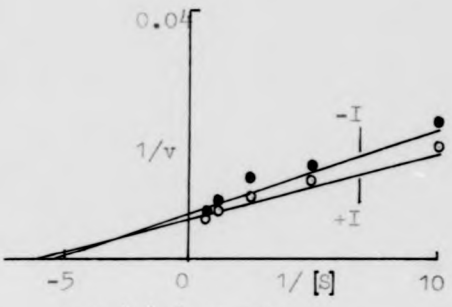
5.3.4.4.1. α-AIB



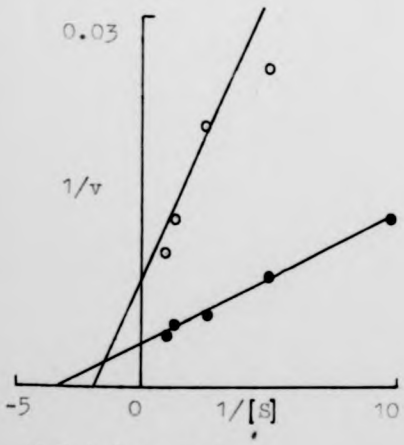
5.3.4.4.2. L-ala



5.3.4.4.3. L-pro



5.3.4.4.4. L-phe



5.3.4.4.5. L-leu

α-AIB + 4 mM cyclo plot omitted (too few data points)

Figures 5.3.4.4.1. - 5.3.4.4.5. Lineweaver-Burk plots of Figures 5.3.4.3.1.- 5.3.4.3.5.

Table 5.3.4.4. Inhibition of ^{14}C-amino acid uptake (@ 0.1mM) by 2mM unlabelled cycloleucine. A comparison between the adult worm and cysticercoid (Adult data from HARRIS & READ, 1968).

^{14}C-amino acid	% Inhibition of uptake	
	Adult worm	Stage V cysticercoid
α-AIB	4.4	93.5
L-alanine	79.2	90.1
L-leucine	93.2	68.6
L-phenylalanine	84.0	0

Table 5.3.4.5. Comparison of K_t and Vmax values in adult H. diminuta and stage V cysticercoids

AMINO ACID	K_t (mM)		V_{max} (µmoles/g/h)		REFERENCES (ADULT ONLY)
	ADULT	CYSTICERCOID	ADULT	CYSTICERCOID	
cycloleucine	0.25	0.28	170	Varies	HARRIS & READ, 1968
∝-AIB	0.55	0.35	-	285	HARRIS & READ, 1968
L-alanine	0.42	0.30	-	184	READ et al., 1963
L-proline	0.44[a]/0.27[b]	0.88	108[b]	362	a= READ et al., 1963 b= KILEJIAN, 1966a
L-leucine	0.21	0.32	-	298	READ et al., 1963
L-phenylalanine	0.14[a]/0.29[b]	0.20	47[b]	132	a= READ et al., 1963 b= PAPPAS & GAMBLE, 1980

L-pro and in the latter case an order of α-AIB>L-ala>L-pro> L-leu>L-phe. This would strongly suggest that cycloleucine, α-AIB, L-alanine, L-proline and L-leucine share at least one uptake locus with which L-phenylalanine, glycine and L-methionine (see Figure 5.3.2.11.) also interact, but that another locus also exists which serves solely for the transport of L-phenylalanine. The specificities of these two loci cannot be completely characterised by this form of analysis, and the more refined method of analysis, which consists of studying all the possible interactions of the six amino acids with each other, which allows this to be achieved forms the content of Section 5.3.5.

5.3.5. Specificity of neutral amino acid transport loci

Figure 5.3.5.1.1. and 5.3.5.1.2. describe the uptake of ^{14}C-α-AIB and ^{14}C-cycloleucine in the presence of unlabelled cycloleucine and α-AIB respectively. Figure 5.3.5.1.2. has been previously shown as Figure 4.4.5. From these graphs it is apparent that the uptake of both compounds is entirely inhibited at an I/S ratio of 75 : 1. This is strong evidence that these two compounds enter the cysticercoid through the same transport loci. In addition, Figure 5.3.5.1.1. suggests that the uptake of ^{14}C-α-AIB by the cysticercoid occurs by mediated processes alone, there being no diffusion component (see Section 4.2.3.). As yet, the loci which these two compounds use to enter the cysticercoid have not been characterised and the remainder of this

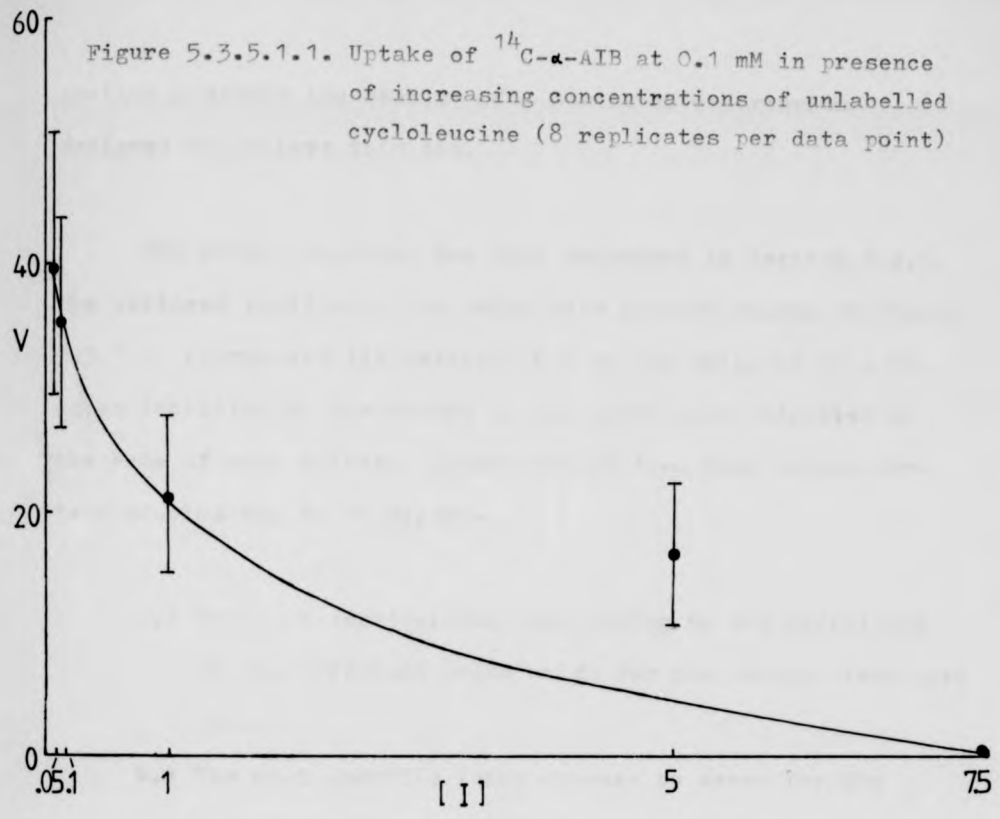


Figure 5.3.5.1.1. Uptake of ^{14}C-α-AIB at 0.1 mM in presence of increasing concentrations of unlabelled cycloleucine (8 replicates per data point)

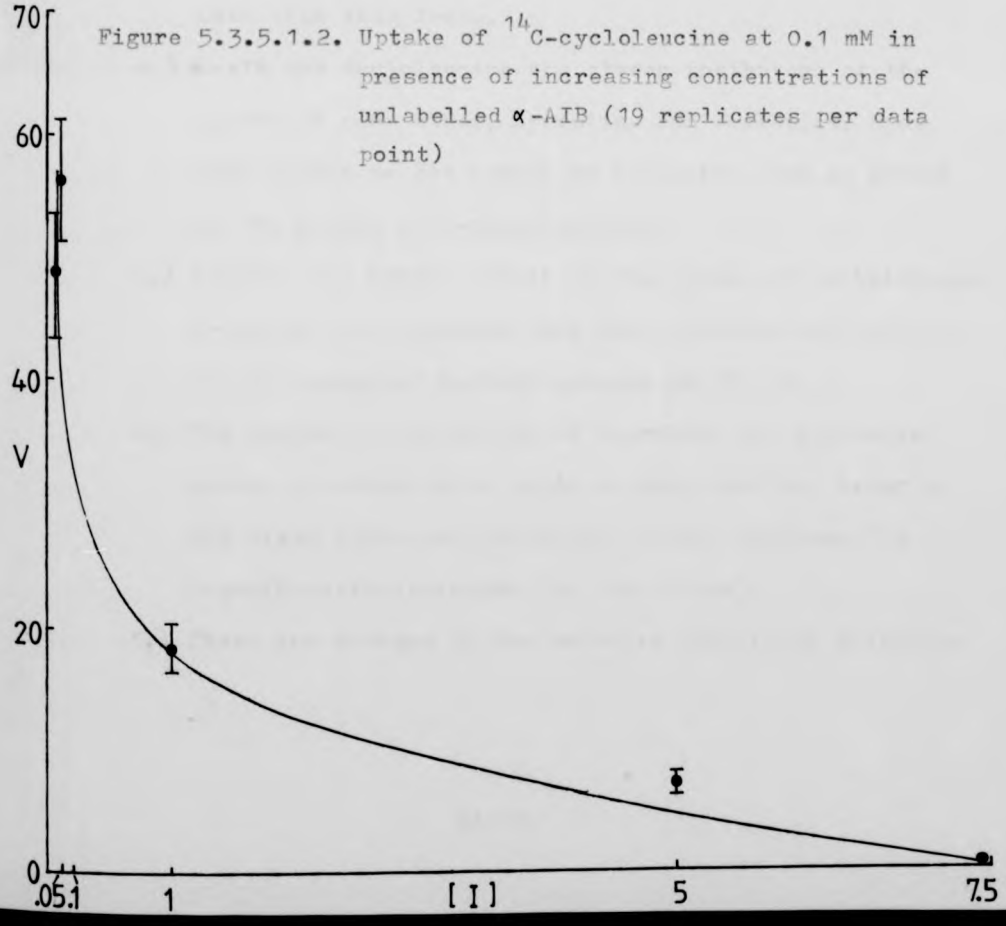


Figure 5.3.5.1.2. Uptake of ^{14}C-cycloleucine at 0.1 mM in presence of increasing concentrations of unlabelled α-AIB (19 replicates per data point)

section presents the results of a series of experiments designed to achieve this aim.

The method employed has been described in section 5.2.6. The vertical position of an amino acid in each column of Figure 5.3.5.2. represents its activity (at an I/S ratio of 10 : 1) as an inhibitor of the uptake of the amino acid indicated at the base of each column. Inspection of these data allows certain conclusions to be drawn:-

a.) There is considerable overlapping in the affinities of the different amino acids for the various transport loci.

b.) The most specific locus appears to serve for the transport of L-phenylalanine. Only L-leucine interacts with this locus.

c.) α-AIB and cycloleucine are strong inhibitors of the uptake of each other, L-proline and L-alanine, have some effect on the uptake of L-leucine, but no effect on the uptake of L-phenylalanine.

d.) Leucine has little effect on the uptake of cycloleucine, L-proline or L-alanine, but has a considerable effect on the uptake of L-phenylalanine and α-AIB.

e.) The degree of inhibition of L-proline and L-alanine uptake by other amino acids is very similar, being in the order (greatest inhibition first) cyclo>α-AIB> L-pro/L-ala>L-leu>L-phe (no inhibition).

f.) There are changes in the relative inhibitory activities

of L-alanine and L-leucine looking from the L-alanine to L-phenylalanine column in Figure 5.3.5.2. The data show a progression from high inhibition by L-alanine, low inhibition by L-leucine on the two left-hand columns, to low inhibition by L-alanine, high inhibition by L-leucine on the two right-hand examples. This 'crossing over' trend of inhibitions suggests the presence of more than one transport locus for the uptake of neutral amino acids, perhaps corresponding to the 'A' and 'L' systems described in the Ehrlich cell by Oxender & Christensen (1963).

A more precise way of examining the relative inhibitory activities of the six amino acids involves the calculation of correlation coefficients. The coefficients describing the interactions of all possible pairs of the six amino acids are shown for each individual amino acid in Figures 5.3.5.3.1.-5.3.5.3.6. and summarised in Table 5.3.5.1. The strength of these interactions has been arbitarily divided into four categories in Table 5.3.5.2. From these two tables, the uptake of the six amino acids appears to be mediated by three transport loci, which have been designated 'Phenylalanine', 'A(alanine)' and 'L(leucine)' (Table 5.3.5.3.). The evidence for this statement will now be summarised.

There is strong evidence for a transport locus existing to mediate the uptake of aromatic neutral amino acids. The uptake of L-phenylalanine is only affected by L-leucine, and the

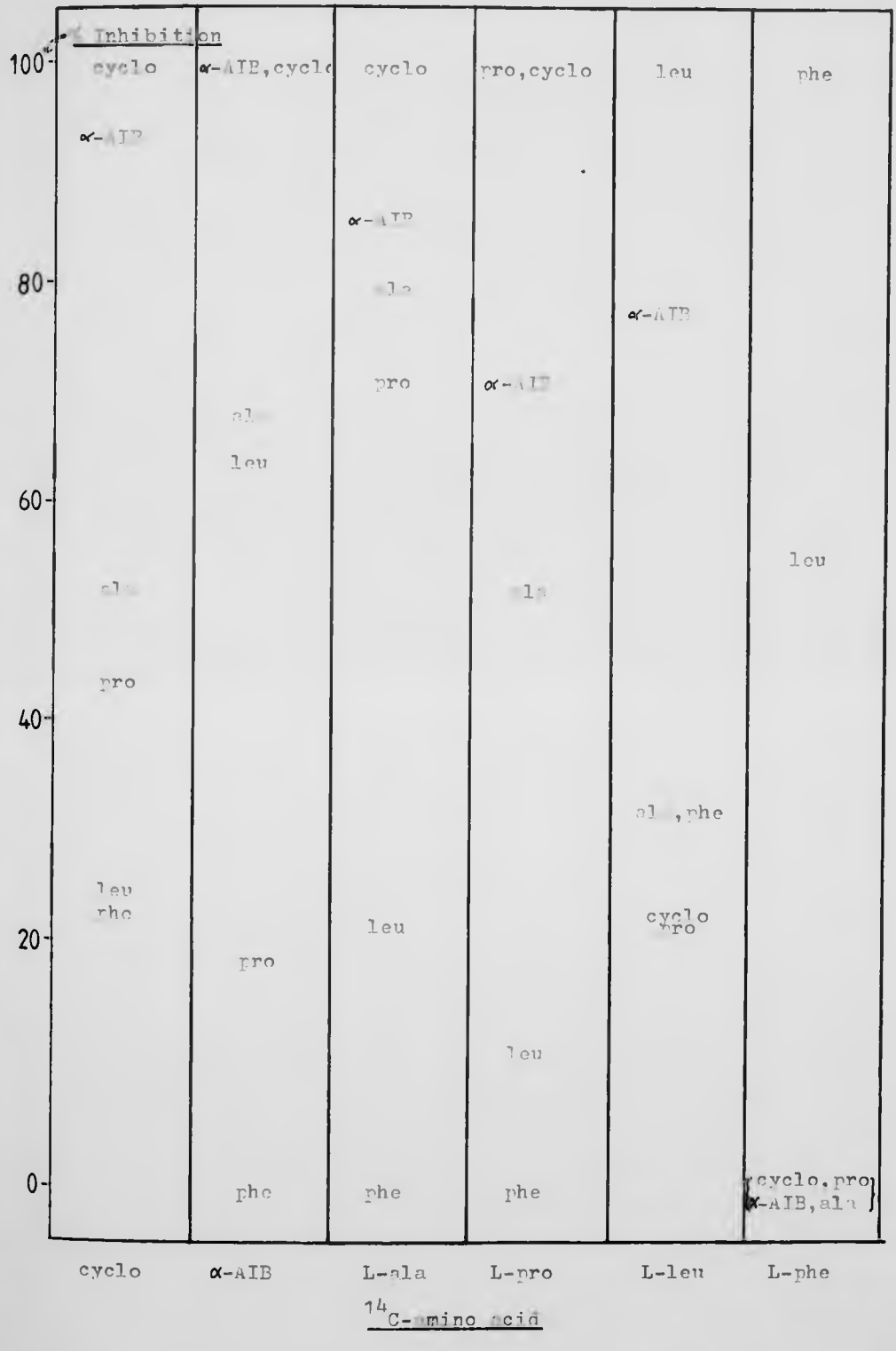
correlation coefficient reveals this to be a 'weak' interaction ($r = 0.31$). No other amino acids interact with this locus. There also appears to be good evidence for a locus which preferentially serves for the transport of aliphatic neutral amino acids. This locus ('A(alanine)') has a wide specificity and is utilised by all six amino acids to a varying degree. L-alanine and L-proline interact strongly with each other, and show similar degrees of interaction with cycloleucine and α-AIB ('medium' interaction) and L-phenylalanine and L-leucine ('weak' interaction). This locus is thus characterised by L-proline and L-alanine uptake. The evidence for a second locus which mediates the uptake of aliphatic neutral amino acids is less strong. This locus, designated 'L(leucine)', and characterised by L-leucine uptake, is only utilised by that compound and L-phenylalanine. L-proline and L-alanine do not interact with this locus, and it is only the weak interactions of cycloleucine and α-AIB with it which set it apart from the 'Phenylalanine' locus.

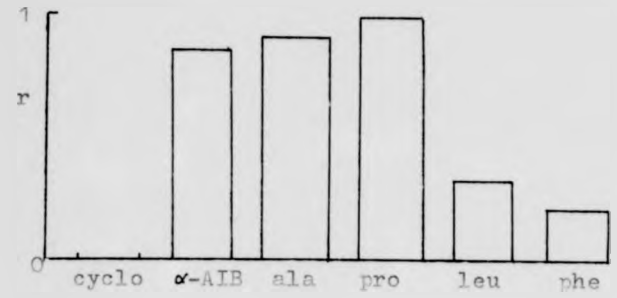
α-AIB and cycloleucine appear to enter through both the 'A(alanine)' and 'L(leucine)' loci, but there is some evidence for the preferential use of one or the other of these loci. α-AIB has a strong inhibitory action on the uptake of L-leucine, and a similar degree of inhibition is noted when the roles of inhibitor and substrate are reversed. In contrast, cycloleucine has little effect on L-leucine uptake, and vice-versa. Both compounds, however, have similar effects on the uptake of L-proline and L-alanine, and no effect on the uptake of L-phenylalanine. From this evidence it would appear likely that α-AIB preferentially

enters through the 'L(leucine)' locus, and cycloleucine through the 'A(alanine)' locus. This difference probably arises from the structural differences between the two molecules.

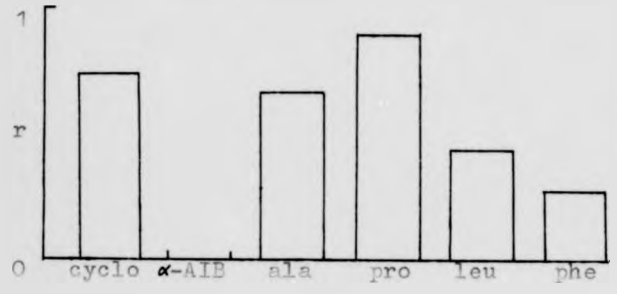
In conclusion, the findings that cycloleucine, α-AIB, L-leucine and L-phenylalanine enter through more than one locus indicate that the K_t values given in Table 5.3.4.1. which describe the uptake of these compounds are composite values, describing the affinities of these compounds for more than one locus.

Figure 5.3.5.2. Inhibition of various ^{14}C-amino acids. [I] = 2 mM, [S] = 0.2 mM. 5 replicates per data point (see text for details).

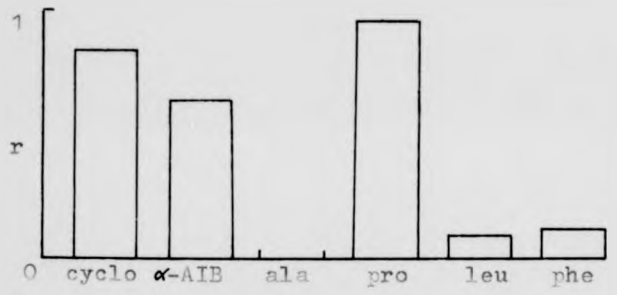




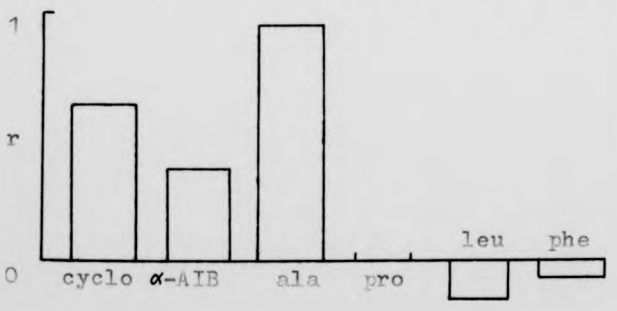
5.3.5.3.1.
cycloleucine



5.3.5.3.2.
α-AIB

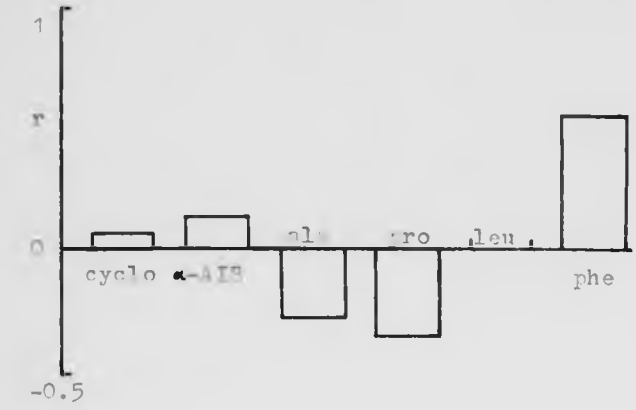


5.3.5.3.3.
L-alanine

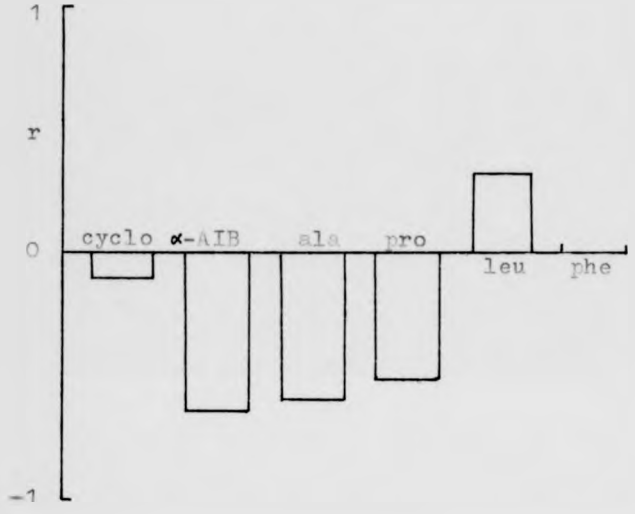


5.3.5.3.4.
L-proline

Figure 5.3.5.3. Plots of correlation coefficients (r) of inhibitions of various amino acids (continues on next page).



5.3.5.3.5.
L-leucine



5.3.5.3.6.
L-phenylalanine

Figure 5.3.5.3. (continued)

Table 5.3.5.1. Summary of results of correlation experiments

y-axis	x-axis					
	cyclo	α-AIB	L-ala	L-pro	L-leu	L-phe
cyclo	-	0.74	0.84	0.62	0.05	-0.09
α-AIB	0.85	-	0.64	0.36	0.15	-0.66
L-ala	0.91	0.69	-	0.94	-0.28	-0.59
L-pro	0.97	0.87	0.97	-	-0.36	-0.53
L-leu	0.32	0.45	0.10	-0.17	-	0.31
L-phe	0.21	0.29	0.13	-0.07	0.54	-

y-axis	x-axis					
	cyclo	α-AIB	L-ala	L-pro	L-leu	L-phe
Cyclo	-	2	2	2	3	4
α-AIB	2	-	2	2	3	4
L-ala	1	2	-	1	4	4
L-pro	1	1	1	-	4	4
L-leu	3	2	3	4	-	3
L-phe	3	3	3	4	2	-

Table 5.3.5.2. Significance of interactions

KEY: 1; r=0.88+ STRONG INTERACTION

2; r=0.44-0.87 MEDIUM INTERACTION

3; r=0.01-0.43 WEAK INTERACTION

4; r=<0 NO INTERACTION

Table 5.3.5.3. Specificity of neutral amino acid uptake loci possessed by stage V cysticercoids of H.diminuta.

Locus	"Phenylalanine"	"A(alanine)"	"L(leucine)"
Strong interactions	phe	ala, pro	leu
Medium interactions	-	cyclo, α-AIB	phe
Weak interactions	leu	leu, phe	cyclo, α-AIB
No interactions	cyclo, α-AIB, pro, ala	-	pro, ala

5.3.6. Studies with N-methylaminoisobutyric acid (N-MeAIB) and 2-amino 2-norbornylcarboxylic acid (2-ANC)

Christensen (1975) has suggested that as the molecules N-MeAIB and 2-ANC show a very high level of specificity for the 'A' and 'L' loci respectively of the Ehrlich cell, they could be used as 'model' amino acids in order to determine whether similar loci exist in other cell types and tissues. Laws & Read (1969) have shown that mono-N-methylation of α-AIB reduces its uptake by tenfold, although the affinity of the analogue for the loci which serve to transport α-AIB is unaffected. In direct contrast to this situation, which concerns the adult H.diminuta, Christensen, Oxender, Liang & Watz (1965) have found that this compound has a greater affinity for the 'A' locus of the Ehrlich cell than the parent compound. Table 5.3.1.3. shows that mono-N-methylation of α-AIB completely abolishes the ability of this compound to interact with the loci possessed by the cysticercoid of H.diminuta which serve to translocate ^{14}C-cycloleucine. There have been no studies concerning the interactions of 2-ANC with helminth parasite amino acid transport loci.

This section examines the uptake of ^{14}C-N-MeAIB and compares it with the uptake of the parent compound. Whether or not there is a high degree of locus specificity shown by both this compound and 2-ANC can be revealed by suitable inhibition studies. From the results of such studies conclusions can be drawn as to whether these two compounds are of value for further

transport studies involving cysticercoids.

Figure 5.3.6.1. shows the time-dependent absorption of 0.2 mM ^{14}C-N-MeAIB by stage V cysticercoids of H.diminuta. Comparison with Figure 5.3.3.1. reveals that the effect of N-methylation is markedly to depress the rate of uptake of α-AIB. Consequently, the degree of accumulation after 30 minutes is also reduced, a concentration ratio of 4.6 being achieved, compared to 23.5 for the parent compound. A kinetic study of the uptake of ^{14}C-N-MeAIB, employing 5 minute incubations, reveals that the rate of uptake is apparently linearly-related to substrate concentration. This result may either be interpreted as showing that the uptake of this compound occurs primarily by diffusion, or that Michaelis-Menten saturation kinetics are not apparent within the concentration range employed (Figure 5.3.6.2.). From the Linewever-Burk plot of the data which has not been corrected for this apparent diffusion component, a mean K_t value of 0.46 mM and a mean V_{max} value of 138 µmoles/g/h can be calculated (Figure 5.3.6.3.). The ranges of these kinetic constants, as calculated by the method described in Section 4.2.2., are 0.35-0.67 mM (K_t) and 105-201 µmoles/g/h (V_{max}). When these values are compared to those obtained with ^{14}C-α-AIB (K_t = 0.35 (standard), 0.22-0.93 mM (range); V_{max} = 285 (standard), 176-752 µmoles/g/h (range)), it is apparent that although N-methylation markedly affects the rate of uptake, the affinity of the analogue for the transport loci which mediate the uptake of the parent compound is not affected.

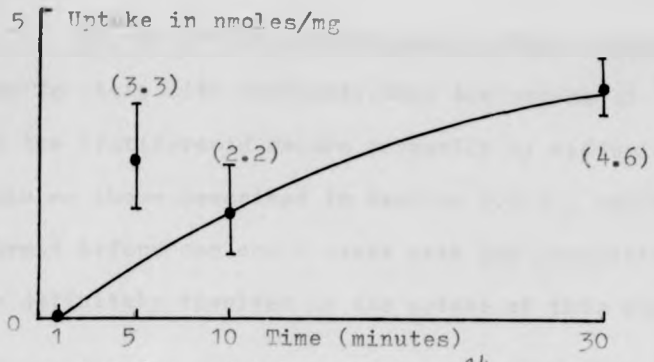


Figure 5.3.6.1. Uptake of 0.2 mM ^{14}C-N-MeAIB. Each data point is the mean of 5 replicates ± S.E. Figures in brackets = concentration ratios.

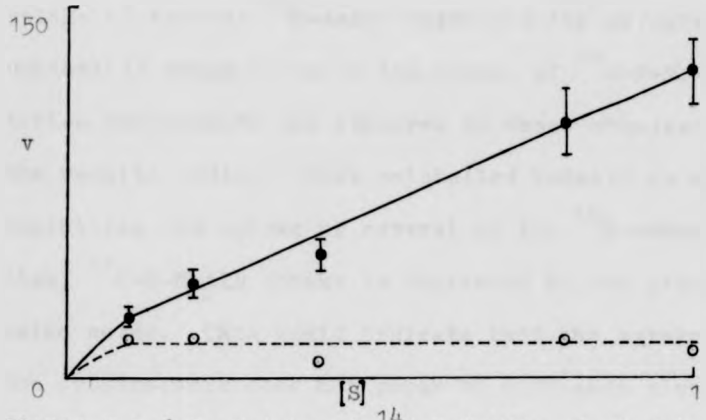


Figure 5.3.6.2. Uptake of ^{14}C-N-MeAIB. Each data point is the mean of 15 replicates ± S.E. —●— = experimental data, ---o--- = data corrected for diffusion.

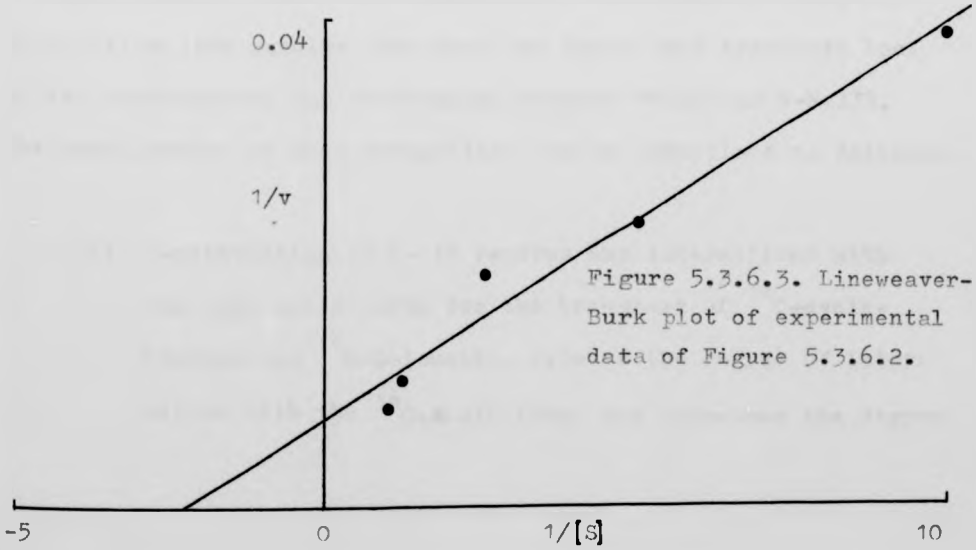


Figure 5.3.6.3. Lineweaver-Burk plot of experimental data of Figure 5.3.6.2.

The analytical methods used in this section do not permit one to state with certainty that the uptake of ^{14}C-N-MeAIB by the cysticercoid occurs primarily by diffusion. Futher tests, such as those described in Section 4.2.3., would need to be performed before one could state with any conviction that diffusion is definitely involved in the uptake of this compound.

Tables 5.3.6.1. and 5.3.6.2. present the results of experiments which show the effects of unlabelled N-MeAIB on the uptake of various ^{14}C-amino acids and the effects of various unlabelled amino acids on the uptake of ^{14}C-N-MeAIB. In both tables the results are compared to those obtained with α-AIB. The results indicate that unlabelled N-MeAIB is capable of inhibiting the uptake of several of the ^{14}C-amino acids tested. Also, ^{14}C-N-MeAIB uptake is depressed in the presence of unlabelled amino acids. This would indicate that the uptake of N-MeAIB by the cysticercoid does not occur by diffusion alone, but that a mediated component of transport exists. However the contributions that these two forms of transport make to the total absorption of this compound cannot be determined by this form of analysis. In addition, the results show that the amino acid transport loci of the cysticercoid can distinguish between α-AIB and N-MeAIB. The consequences of this recognition can be summarised as follows:-

i) N-methylation of α-AIB removes any interactions with the loci which serve for the transport of ^{14}C-cycloleucine and ^{14}C-L-leucine, reduces the degree of interaction with the ^{14}C-α-AIB locus and increases the degree

Table 5.3.6.1. Effects of 2mM unlabelled N-MeAIB on the uptake of various ^{14}C-labelled amino acids (@ 2mM) by cysticercoids of H.diminuta

^{14}C-amino acid	% Inhibition	
	by N-MeAIB	by α-AIB
cyclo	1.0*	87.6
α-AIB	29.5	100.0
L-ala	60.5	86.3
L-pro	100.0	70.9
L-leu	0	77.2
L-phe	7.3*	0

5-10 replicates per experiment

*denotes result NOT significant at p = 0.05

Table 5.3.6.2. Effects of various unlabelled amino acids (2 2mM) on the uptake of 0.2mM ^{14}C-N-MeAIB by cysticercoids of H.diminuta

Inhibitor	% Inhibition	
	of N-MeAIB	of α-AIB
N-MeAIB	59.1	29.5
α-AIB	56.8	100.0
cyclo	58.5	100.0
L-ala	22.9	67.4
L-pro	54.4	16.6
L-leu	21.1	63.2
L-phe	55.5	2.3*

5-10 replicates per experiment

*denotes result NOT significant at p = 0.05

Table 5.3.6.3. Correlation coefficients describing interactions between various amino acids and N-MeAIB/α-AIB

Amino acid	α-AIB	N-MeAIB
cyclo	0.74	-0.01
α-AIB	-	-0.34
L-leu	0.45	-0.10
L-ala	0.69	0.40
L-phe	0.29	0.27
L-pro	0.87	0.49

Table 5.3.6.2. Effects of various unlabelled amino acids (@ 2mM) on the uptake of 0.2mM ^{14}C-N-MeAIB by cysticercoids of H.diminuta

Inhibitor	% Inhibition	
	of N-MeAIB	of α-AIB
N-MeAIB	59.1	29.5
α-AIB	56.8	100.0
cyclo	58.5	100.0
L-ala	22.9	67.4
L-pro	54.4	16.6
L-leu	21.1	63.2
L-phe	55.5	2.3*

5-10 replicates per experiment

*denotes result NOT significant at p = 0.05

Table 5.3.6.3. Correlation coefficients describing interactions between various amino acids and N-MeAIB/α-AIB

Amino acid	α-AIB	N-MeAIB
cyclo	0.74	-0.01
α-AIB	-	-0.34
L-leu	0.45	-0.10
L-ala	0.69	0.40
L-phe	0.29	0.27
L-pro	0.87	0.49

Table 5.3.6.4. Inhibition of 0.2mM ^{14}C-amino acid uptake by unlabelled 2mM 2-amino-2-norbornancarboxylic acid (2-ANC)

^{14}C-amino acid	% Inhibition
cyclo	70.5
α-AIB	77.2
N-MeAIB	78.1
L-ala	62.0
L-pro	28.5
L-leu	69.5
L-phe	51.4

Each result is the mean of 5 replicates.

of interaction with the ^{14}C-L-proline locus. There is little change in the degree of interaction with the ^{14}C-L-alanine and ^{14}C-L-phenylalanine loci.

ii) Unlabelled α-AIB, cycloleucine, L-alanine and L-leucine inhibit the uptake of ^{14}C-N-MeAIB less than ^{14}C-α-AIB, whereas unlabelled N-MeAIB, L-proline and L-phenylalanine are more effective inhibitors of ^{14}C-N-MeAIB uptake .

By calculating the correlation coefficients (see Section 5.2.6.) the strengths of the interactions between N-MeAIB and the various amino acids utilised in this study can be determined. The values of such coefficients are summarised, and compared to those obtained with α-AIB, in Table 5.3.6.3. From these results it is apparent that the primary consequence of N-methylation is to remove the interactions of α-AIB with the loci which transport cycloleucine, L-leucine and α-AIB. Figure 5.3.5.3. shows that these three compounds enter the cysticercoid through both the L(leucine) and A(alanine) loci. However, N-MeAIB still interacts with the loci transporting L-proline and L-alanine, and these compounds enter the cysticercoid solely through the A(alanine) locus. The effect of N-methylation, then, on the transport of α-AIB would seem to be a removal of any interaction with the L(leucine) locus, and a shift toward the A(alanine) locus. In this respect the amino acid transport loci of the cysticercoid resemble those of the Ehrlich cell (Christensen et al., 1965). The interactions with the A(alanine) locus are, however, weaker than those shown by α-AIB. This may explain the reduced rate of transport of the analogue per

unit time (Figure 5.3.6.2.).

Table 5.3.6.4. depicts the effects of 2-ANC on the uptake of a number of ^{14}C-labelled amino acids. Although all of the amino acids employed in this particular study are capable of utilising both A(alanine) and L(leucine) loci, one could reasonably expect that such large inhibitions of L-alanine, α-AIB, cycloleucine and N-MeAIB uptake (62.0%, 77.2%, 70.5% and 78.1% respectively, for compounds of which 60-100% entry is through the A(alanine) locus - see Figure 5.3.5.2. and Table 5.3.6.2.) would not be caused by a compound claimed to be highly specific for loci resembling the 'L' locus of the Ehrlich cell. Such results may reflect the absence of such loci in the cysticercoid of M.diminuta, although the relatively strong inhibitions of L-leucine and L-phenylalanine uptake and the weak inhibition of L-proline uptake suggest that some of the criteria for the presence of an 'L'-type locus as evidenced by the effects of 2-ANC are being met.

This brief study has shown that N-MeAIB and 2-ANC are of some use in cysticercoid transport studies. The use of N-MeAIB has revealed further differences in the characteristics of the amino acid transport loci of cysticercoid and adult and has suggested that an 'A'-type locus may exist in the cysticercoid. 2-ANC has never been used in studies with the adult worm, but further studies with this compound should provide evidence for the presence or absence of an 'L'-type locus in the cysticercoid.

5.4. Discussion

The results presented in Sections 5.3.1. and 5.3.2. suggest that the mechanisms possessed by Stage III and Stage V cysticercoids which serve for the transport of ^{14}C-cycloleucine have several distinguishing features:-

a.) They are highly specific for neutral amino acids (carbohydrates, a fatty acid, betaine, L-lysine and L-glutamate do not interact).

b.) Neutral amino acids interacting with these systems do so in a competitive fashion.

c.) The integrity of the amino group, its position on the carbon chain and the structure of the side chain all contribute to the inhibitory action of a competing amino acid.

d.) There is some evidence that stereospecificity towards the D- and L- isomers of competing amino acids exists.

e.) They bear many similarities to the ^{14}C-cycloleucine transport loci posses ed by adult H.diminuta.

The ineffectiveness of molecules other than neutral amino acids or their derivatives as inhibitors of the uptake of neutral amino acids by tapeworms is well documented. Arme & Coates (1973), using the Stage V cysticercoid of H.diminuta, found that glucose, galactose, fructose, lactose, sodium acetate, sodium stearate and sodium lactate had no effect on the uptake of ^{14}C-α-AIB by this parasite. Kilejian (1966a) found that

glucose had no effect on the uptake of ^{14}C-L-proline by adult *H.diminuta*, but pre-incubation with this compound markedly depressed the subsequent rate of uptake of ^{14}C-L-proline in the absence of glucose. Kilejian interpreted this latter result by suggesting that glucose interfered with metabolism in such a way as to act as an "allosteric" inhibitor of proline transport. However, pre-incubation in glucose had no effect on the uptake of ^{14}C-cycloleucine by various tissues of *Ascaris* in the absence of glucose (Harris, Talent & Podger, 1972). Glucose was also found to have no effect on the transport of ^{14}C-lysine, ^{14}C-phenylalanine or ^{14}C-methionine (Pappas & Read, 1973) or ^{14}C-valine (Haynes & Taylor, 1968) by *T.crassiceps* metacestodes, ^{14}C-cycloleucine by *S.mansoni* (Isseroff, Ertel & Levy, 1976) and ^{14}C-leucine by *Cotugnia digonopora* (Roy & Srivastava, 1981).

Table 5.3.1.1. indicates that the loci involved in the transport of cycloleucine are also involved in the transport of a wide variety of other neutral amino acids. Numerous examples of the broad specificity of helminth neutral amino acid transport loci are summarised by Pappas & Read (1975) and Podesta (1982). It is interesting to note that there is some similarity between the rank orders of the solubility of amino acids in water and their effectiveness as inhibitors of ^{14}C-cycloleucine transport (Table 5.4.1.). This may be due to the effects of stationary water layers on the rates of transport of these compounds.

The recognition of a molecule by the cycloleucine transport

Table 5.4.1. Solubility of amino acids in water compared to their effectiveness as inhibitors of 0.1 mM ^{14}C-cycloleucine uptake by Stage V cysticercoids of H.diminuta

amino acid	Solubility[a]	% Inhibition[b]
L-pro	166.2	42.4
gly	25.0	59.7
L-ala	16.7	51.1
L-val	8.6	51.1
DL-ser	5.0	82.4
L-ile	4.1	29.4
DL-met	3.4	27.7
L-phe	2.4	22.2
L-trp	1.1	36.0
L-glu	0.9	0
L-cys	0.01	13.4

[a] = Solubility expressed as grams amino acid per 100 grams pure water at 25°C (values from CRC Handbook of Chemistry and Physics, 56th Edition (1975-6))

[b] = Values taken from Table 5.3.1.1. All for L-isomers

Rank Orders (greatest first)

Solubility: pro>gly>ala>val>ser>ile>met>phe>leu>trp>glu>cys

% Inhibition: ser>gly>ala>val>pro>try>ile>met>leu>phe>cys>glu

loci appears to be dependent on the possession of certain structural features. A primary requirement for transport by such loci is the presence of an intact amino group. The results presented in Table 5.3.1.3. show that methylation of the amino group of α-AIB completely removes the inhibitory action of this molecule on the uptake of ^{14}C-cycloleucine. Equally important is the position of this group relative to the carboxyl group (Table 5.3.1.3.). Read et al. (1963), using adult H.diminuta, showed that similar requirements exist for the absorption of methionine. As in this study, substitution in the amino group of the methionine molecule completely abolished the reactivity of the analogue with the methionine locus, and moving the amino group from the α- to the β- to the γ- carbon atom of the AIB molecule reduced the inhibitory effectiveness of this compound as an inhibitor of methionine transport from 89% to 33% to 0% respectively. Both Laws & Read (1969), and the current study (see Figure 5.3.6.1.), using the adult and Stage V cysticercoid of H.diminuta respectively, found that N-methylation of α-AIB markedly reduced the uptake of this compound, and that the effectiveness of this analogue as an inhibitor of various ^{14}C-amino acids was different to that of unaltered α-AIB (see Table 5.3.6.1.).

The initial recognition of a suitable molecule by a neutral amino acid transport locus, then, would appear to be dependent upon the possession by that molecule of an intact amino group, and its position relative to the carboxyl group. The free hydrogen atom borne by most amino acids does not seem to be

recognised, or it is unlikely that either AIB or cycloleucine, which do not possess this feature, would be transported. The degree of interaction with the loci responsible for the transport of cycloleucine appears to be dependent on the structure of the amino acid side chain. Effectively, there are four separate side chain groups, with respect to their effectiveness as inhibitors of ^{14}C-cycloleucine uptake. The most effective inhibitors of ^{14}C-cycloleucine transport are the group of amino acids bearing a single oxygen atom within their side chain. This group consists of (% inhibition in brackets - data from Table 5.3.1.1.) serine (82.4), glutamine (77.6), asparagine (72.6) and threonine (61.1). Within this group, reactivity with the cycloleucine loci is diminished by a lengthening of the hydrocarbon chain, or the possession of a methyl group (i.e. threonine). The second group are characterised by the presence of an aliphatic hydrocarbon chain. The simplest of these is glycine (59.7), and reactivity declines as the chain lengthens viz: alanine (51.1), valine (51.1), proline (if considered to be a chain of three carbon atoms bent around on themselves - 42.4), isoleucine (29.4) and leucine (24.7). Logically, the next compound in this sequence would be lysine, but this does not interact with these loci. This may be due to the overall length of the hydrocarbon side chain, rather than the possession of a terminally-located amino group, as both glutamine and asparagine are highly effective inhibitors of cycloleucine transport and both bear such a group in a terminal position. The third group comprises those molecules bearing a sulphur atom, represented by L-methionine (27.7) and cysteine

(13.4). Reactivity within this group may be dependent on the degree of masking of the sulphur atom, rather than the length of the hydrocarbon chain. Cysteine only masks this atom with a single hydrogen, but bears a short hydrocarbon chain, whereas although the hydrocarbon chain of the methionine side chain is longer than that of cysteine, a methyl group covers the sulphur atom. It is not suggested that the sulphur atom is recognised by the loci, but its presence appears to hinder the recognition of the side chain. The final group is comprised of those compounds with large aromatic side chains, phenylalanine (22.2) and tryptophan (36.0). No explanation can be proffered for the greater inhibitory action of tryptophan which bears a far bulkier side chain than phenylalanine. One compound, α-AIB (87.6), does not fit into any of these four groups. Its unique structure may be sterically favourable to the loci responsible for its transport.

A structural hypothesis has been offered to explain the ineffectiveness of lysine as an inhibitor of cycloleucine uptake. One could propose with equal vigour that L-glutamic acid has no inhibitory action on cycloleucine transport due to its possession of a second oxygen atom within its side chain. However, at a pH of 6.0, glutamic acid would bear a net negative, and lysine a net positive charge, and it is possible that charge, not steric hindrance, is the dominant factor in determining these compounds degree of interaction with the cycloleucine transport loci. The pH of Tenebrio haemolymph is not known, but the haemolymph of a wide variety of insects has been found to be slightly acid (Wigglesworth, 1972).

Further inhibition experiments revealed that the order of certain neutral amino acids as competitive inhibitors of cycloleucine uptake was α-AIB>cyclo>L-ala>gly>L-pro>L-leu>L-met>L-phe>D-met. This order mirrors the results presented in Table 5.3.1.1., with the exception of those concerning the inhibitory effectiveness of L-alanine. This may be explained by the similarities between the structure of this compound and α-AIB, which only differ by the presence of a hydrogen atom by alanine and a methyl group by α-AIB at the α-carbon position. These similarities are more strongly expressed by calculation of the inhibition constants than by comparing percentage inhibitions at a single I:S ratio.

The similarities between the cycloleucine transport loci of stages III and V cysticercoids and adult H.diminuta have already been noted (Table 5.3.1.6.), and this may be a reflection of the same structural criteria having to be met by molecules entering the parasite through these loci. This strongly suggests that these loci are the same in all three developmental stages. There has only been one other study, except for that of Harris & Read (1968), involving the absorption of cycloleucine by a helminth parasite in the presence of competing molecules. This is the survey of Isseroff et al. (1976), who found the percentage inhibition of 0.1 mM ^{14}C-cycloleucine uptake by adult S.mansoni in the presence of unlabelled 10 mM alanine, aspartate, cycloleucine, glutamate, leucine, phenylalanine, serine and valine to be 0,0, 45, 0, 45, 39, 27, and 25% respectively. The I:S ratio employed in this particular study was 100:1, compared with the

ratio of 20:1 used in this study and that of Harris & Read (1968). A comparison of the rank orders of the inhibitory effectiveness of the aforementioned amino acids upon the uptake of ^{14}C-cycloleucine gives an order of (most effective inhibitor first) cyclo>ala>phe>leu>glu (0) for H.diminuta cysticercoids, and cyclo=leu>phe>ala=glu (0) for S.mansoni. This strongly suggests that the structural criteria which must be met by an amino acid to be recognised by the cycloleucine transport loci differ considerably in these two species. Haynes & Taylor (1968), have also found that certain structural criteria must be met for neutral amino acids to enter the interior of T.crassiceps metacestodes via the valine and methionine uptake loci of this parasite. Proline cannot enter through these loci and the compounds causing the greatest inhibitions of valine and methionine uptake were those amino acids with long, unbranched sidechains such as methionine, threonine, asparagine and glutamine.

Pappas & Read (1975) have claimed that the amino acid transport systems of cestodes are unique when compared with those of mammals in that they show either an equal affinity for both stereoisomers of a particular amino acid or an increased affinity for the D-form. Mammalian systems, in contrast, show a preference for the L-form. However, a critical examination of the available data does not support the claim of Pappas & Read. Although it is true that adult H.diminuta shows an equal affinity towards both stereoisomers of methionine (Read et al., 1963), there is a tendency for this affinity to

vary with both the species of cestode being used and the amino acid under study. Equal affinity is exemplified by the studies of Senturia (1964) and Pappas & Gamble (1980), involving adult H.citelli and H.diminuta respectively. Senturia found that the L-methionine transport loci of H.citelli cannot distinguish between the D- and L-stereoisomers of methionine, and Pappas & Gamble showed a similar lack of recognition when unlabelled D- or L-tryptophan, tyrosine or phenylalanine were used to inhibit the uptake of the ^{14}C-labelled forms of these compounds. Arme & Coates (1973), using the cysticercoid of H.diminuta, found that at an I:S ratio of 10:1, the uptake of α-AIB was inhibited 86% by D-alanine and 93% by L-alanine, 81% by D-methionine and 91% by L-methionine, and 61% by D-valine and 49% by the L-isomer. Whether these differences are significant is questionable, but this current study has clearly shown that the cysticercoid of H.diminuta can distinguish between the D- and L-stereoisomers of methionine. Table 5.3.1.1. shows, that at an I:S ratio of 10:1, the uptake of ^{14}C-cycloleucine is inhibited 27.7% by L-methionine but is not significantly affected in the presence of D-methionine. This dichotomy is further clarified by a consideration of the appropriate K_i values: 3.52mM for L-methionine and 14.64mM for D-methionine. These compare to a K_t(cycloleucine) value of 0.28mM (see Table 5.3.2.1.). There have been no previous studies involving the calculation of K_i values when stereospecificity has been investigated in cestodes. The data concerning T.crassiceps metacestodes shows a wide range of variation. Haynes & Taylor (1968) found that the uptake of L-methionine was inhibited 58%, 47% and

32% by D-, DL- and L-alanine respectively, While Pappas et al. (1973), also using L-methionine, reported no differences between the effectiveness of D- and L-methionine as inhibitors, but noted a clear preference for the D-isomers of valine and alanine. In the same study, the loci responsible for the transport of L-lysine showed a clear preference towards the L-isomers of lysine and arginine.

Table 5.3.1.6. suggests that there are considerable similarities between the cycloleucine transport loci possessed by the cysticercoid and adult of H.diminuta. The possible significance of this similarity, which is strongly reflected in the virtually identical K_t values for cycloleucine uptake by the two developmental stages, will be considered in Section 7.

When the uptake by the cysticercoid of amino acids other than cycloleucine is considered, it is found that all of the compounds tested (α-AIB, L-leucine, L-alanine, L-phenylalanine, L-proline) are rapidly absorbed and accumulated. In common with previous studies involving the accumulation of amino acids by adult H.diminuta, the cysticercoid appears to be limited with regard to the amount of a specific amino acid which it can accumulate. The uptake of 0.2mM ^{14}C-L-leucine appears to have reached a steady state after between 10 and 30 minutes incubation, but all of the other compounds (including cycloleucine) have not reached equilibrium aftwr 30 minutes. Only eight amino acids have been shown to be actively accumulated by adult H.diminuta (see Introduction, Section 1). After 30 minutes

incubation in 0.1mM ^{14}C-L-proline, cycloleucine or L-phenylalanine, concentration ratios of 40, 35 and 70 respectively were achieved by the adult worm (Kilejian, 1966b; Harris & Read, 1968; Pappas & Gamble, 1980). These values compare with concentration ratios of 32, 25 and 14 in the cysticercoid after 30 minutes incubation in 0.2mM solutions of the same three amino acids. As a general rule, the quantity of amino acid absorbed per unit time by a tapeworm increases as the substrate concentration increases (see, for example, Fig. 2 in Kilejian, 1966b). This law would also appear to hold good for the cysticercoid of H.diminuta (see Figs. 4.3.3. and 4.3.4.). At first sight, it would appear that the cysticercoid cannot form so large a pool of proline, cycloleucine or phenylalanine within its tissues as the adult worm. This may be due to differences in the rate of efflux or degree of internal compartmentalisation between the two stages, but the results of Section 6 suggest that the difference may reside in the comparative rates of amino acid incorporation into protein. Table 6.3.3.1. shows that the cysticercoid can incorporate more free pool L-leucine into protein per unit mass per hour than the adult worm, and this may also be the case in the incorporation of other amino acids. Such an explanation cannot, however, explain the disproportionate accumulation of cycloleucine by the two stages in the life cycle as this compound cannot be incorporated into protein.

The typical saturation isotherms of Figs. 5.3.4.1.1.-5.3.4.1.6. suggest that the uptake of α-AIB, L-leucine, L-alanine, L-phenylalanine and L-proline is by mediated transport.

Arme & Coates (1973) reached a similar conclusion for the transport of α-AIB by the cysticercoid as did Harris & Read (1968) for the adult worm. The study of Read et al. (1963) with adult H.diminuta claimed that the uptake of the four other amino acids listed above was by mediated transport alone, although consequent studies have shown that L-phenylalanine and L-proline are absorbed, at least in part, by diffusion (Pappas & Camble, 1980; Kilejian, 1966a). Further evidence for the mediated uptake of these five compounds is provided by the data of Tables 5.3.5.2. 5.3.5.3., which show that the uptake of each of them may be inhibited by at least one structurally similar compound. In the cases of α-AIB, L-leucine, L-proline and L-alanine, the inhibition of uptake by cycloleucine was shown to be competitive (Figs. 5.3.4.1.1.-5.3.4.1.5.). The demonstration of accumulation (Table 5.3.3.1.) suggests that the mediated transport of all five compounds is active.

Table 5.3.4.5. gives a comparison of the K_t and V_{max} values describing the uptake of the five aforementioned amino acids (plus cycloleucine) by the adult and cysticercoid stages of H.diminuta. When the range of each K_t value obtained with the cysticercoid is taken into consideration (see Table 5.3.4.1.), the values describing the uptake of cycloleucine, α-AIB, L-alanine, L-leucine and L-phenylalanine may be considered to be very similar in both developmental stages. In addition, K_t(α-AIB) obtained in this study is very similar to the value obtained by Arme & Coates (1973). However, the K_t value describing L-proline uptake by the cysticercoid is considerably

greater (denoting a lowered affinity) than that calculated for the adult worm. Proline is the most abundant amino acid in Tenebrio haemolymph (Table 7.2.), and such a lowered affinity may be a device to prevent flooding of the cysticercoids internal tissues with this compound. In addition, it may also stop proline blocking the uptake of other amino acids by the cysticercoid.

From the data presented in Figure 5.3.5.2., comparisons can be drawn between the characteristics of the loci serving for the transport of L-alanine, L-proline, L-leucine and L-phenylalanine by the adult and cysticercoid of H.diminuta. Such a comparison has been made in Table 5.4.2., which suggests that, despite the range of I:S ratios employed, there are considerable similarities between the loci responsible for the transport of L-alanine, L-leucine and especially L-proline in both developmental stages. There are some notable differences, however, particularly with regard to phenylalanine. The L-alanine locus of the cysticercoid is not utilised by phenylalanine, and a similar situation exists for the L-proline loci. However, the uptake of L-proline by the adult worm is only inhibited 4% in the presence of phenylalanine, and such a result may be statistically identical to zero inhibition. The L-leucine loci appear similar in both stages. Like those of the adult worm, the L-phenylalanine loci possessed by the cysticercoid are utilised by both phenylalanine and leucine, but unlike the adult worm, are not used by alanine or proline.

Table 5.4.2. % Inhibition of L-alanine, L-leucine, L-phenylalanine and L-proline uptake by these compounds acting as inhibitors. Comparison between adult and cysticercoid of *H.diminuta* (% Inhibition in brackets)

L-ALANINE		L-LEUCINE		L-PROLINE	
cyst[a]	adult[b]	cyst[a]	adult[b]	cyst[a]	adult[b,c]
ala (79.4)	ala (55)	leu (100)	leu (74)	pro (100)	pro (71)
pro (71.4)	pro (54)	ala (76.2)	ala (52)	ala (53.1)	ala (68)
leu (21.0)	leu (36)	phe (46.0)	pro (32)	leu (10.8)	leu (11)
phe (0)	phe (30)	pro (22.4)	phe (28)	phe (0)	phe (4)

L-PHENYLALANINE		
cyst[a]	adult[b]	adult[d]
phe (100)	phe (67)	phe (90)
leu (54.2)	leu (65)	leu (84)
ala/pro (0)	ala (46)	ala (73)
	pro (27)	

a = data from this study. I:S ratio = 10:1

b = data from MacInnis et al. (1976). I:S ratio = 5:1

c = In addition, Kilejian (1966a) noted that L-proline uptake is inhibited by proline, alanine and leucine

d = data from Pappas & Gamble (1980). I:S ratio = 100:1

In a previous study concerning the uptake of α-AIB by the cysticercoid of H.diminuta, Arme & Coates (1973), using an I:S ratio of 20:1, obtained the following percentage inhibitions of α-AIB uptake; ala (92.5%)>cyclo (87.7%)>leu (68.8%). The equivalent results obtained in this study at an I:S ratio of 10:1 were cyclo (100%)>ala (67.4)>leu (63.2). Thus the current study has found a greater inhibitory effect of cycloleucine upon the uptake of α-AIB, and a reduced inhibitory effect of alanine. It is possible that these differences arise from the differences in methodology between the two studies. In particular, this study has shown that both the age of the cysticercoid (Section 4) and the infection density of the cysticercoids within the experimental host (Section 2.4.7.) affect the uptake characteristics of ^{14}C-cycloleucine. Neither of these factors were taken into account by Arme & Coates.

The inhibition studies detailed in Sections 5.3.4. and 5.3.5. indicate that neutral amino acid transport by the cysticercoid occurs through several loci. Table 5.3.5.3. indicates that three such loci exist for the uptake of the six amino acids studied in these sections. It is important to stress, however, that none of these loci are specific for any one amino acid, although the Phenylalanine locus is only utilised by this compound, and, to a minor extent, L-leucine. There are numerous examples in the literature of the wide specificities of cestode amino acid transport loci (see Pappas & Read, 1975). Figure 5.4.1. is a comparison of the three cysticercoid loci with the serine, phenylalanine and leucine loci of the adult

Strength of interaction (r value)	Locus ADULT (TOP) Leucine	Serine	Phenylalanine
Strong (0.88+)	leu	ala, pro	phe
	leu	ala, pro	phe
Medium (0.44+)	ala,pro,phe	leu	leu
	phe	cyc,α-AIB	-
Weak (0+)	-	phe	ala
	cyc,α-AIB	leu,phe	leu
None (>0)	-	-	pro
	pro,ala	-	α-AIB,cyc,ala,pro
	L(leucine)	A(alanine)	Phenylalanine
	CYSTICERCOID (BOTTOM)		

Figure 5.4.1. Comparison of specificities of neutral amino acid transport loci in adult and cysticercoid of *H.diminuta*. Adult; each result based on 15 degrees of freedom (d.o.f), I:S - 5:1. Cysticercoid: each result based on 4 d.o.f, I:S = 10:1

H.diminuta.(see Fig. 1.8.). An examination of this reveals close similarities between the A(alanine) locus and the serine locus, but several differences between the other two loci. The least specific locus is the A(alanine) system. The degrees of interaction of proline, alanine, leucine and phenylalanine with this locus are of similar magnitude to the equivalent serine locus of the adult worm. Harris & Read (1968) found that α-AIB and cycloleucine entered the adult H.diminuta through two loci, one of which could be inhibited by proline, and was considered by these authors to resemble the A (alanine-preferring) site of the Ehrlich cell (Oxender & Christensen, 1963), and the other of which could be inhibited by phenylalanine. The A-type locus probably corresponds to the serine locus, distinguished by MacInnis et al. (1976), and accounts for 30-40% of the total cycloleucine transport. Thus it is likely that the A(alanine) locus of the cysticercoid and the serine locus of the adult are essentially the same. In contrast, the phenylalanine transport system of the cysticercoid differs considerably from that of the adult. Harris & Read (1968) found that cycloleucine, α-AIB and proline all interacted with the systems responsible for the uptake of phenylalanine by the adult worm, and the more extensive study of adult H.diminuta aromatic amino acid transport performed by Pappas & Gamble (1980) showed that alanine also interacts with the postulated pair of loci which serve for the transport of L-phenylalanine. A further point concerning the transport of L-proline is that this current study is in agreement with the findings of Kilejian (1966a) who found that, at low concentrations, L-proline enters the adult H.diminuta

through a single locus. This disagrees with the conclusions of Harris & Read (1968) who speculate that the entry of this compound into the adult worm is via two loci. Finally, differences are also apparent when the characteristics of the adult leucine and cysticercoid L(leucine) loci are compared. Proline and alanine do not interact with the cysticercoid system, but are recognised by the adult locus. Obviously firm conclusions cannot be drawn from the interactions of a mere six amino acids as investigated in this study compared to the fifteen compounds utilised by McInnis et al. (1976), but it would appear that there are clear differences between the loci possessed by the cysticercoid and adult stages of H.diminuta. With no knowledge of the metabolism of the cysticercoid it is almost impossible to speculate on the possible reasons for these differences, but it is conceivable that they may be connected with the differing patterns of development, and the widely-differing physico-chemical environments, experienced by the two stages of the cestodes life cycle.

6. PROTEIN SYNTHESIS STUDIES

6.1. Introduction

The protein metabolism of helminths in general has been reviewed by von Brand (1979) and Barrett (1981) and that of cestodes in particular by Harris (1983); the latter author has stated that this is an almost totally neglected field. A possible reason for this is that protein synthesis in the few cestode species examined appears to be similar to that of mammals (Agosin & Repetto, 1967; Agosin & Naquira, 1978; Naquira, Paulin & Agosin, 1977; Parker & MacInnis, 1977) and is thus an unsuitable target for chemotherapeutic agents. In adults the neck region of the strobila is involved in proglottis production, although Bolla & Roberts (1971) found that the rate of protein synthesis in adult H.diminuta was highest in gravid proglottides, and dependent upon carbohydrate availability. Harris & Read (1969) confirmed the importance of carbohydrate, and also carbon dioxide, in ^{14}C-lysine incorporation into protein by H.diminuta; they also demonstrated that the rate of lysine incorporation was linear over a 2h period, dependent upon lysine concentration and could be inhibited by 2,4-dinitrophenol.

There have been no comparable studies with the cysticercoid of H.diminuta although Prescott & Voge (1959), using autoradiography, have shown that the rate of RNA synthesis is highest in the cysticercoid tegument and the body-tail junction of the metacestode. The incorporation of leucine into protein has also been demonstrated in the metacestode of T.crassiceps by Culbreth,

Esch & Kuhn (1972), and these authors showed that there was an inverse relationship between parasite biomass and leucine incorporation.

In this study, the ability of H.diminuta cysticercoids to incorporate amino acids into protein is investigated, together with certain other factors that might affect this process.

6.2. Materials and Methods

Cysticercoids of known age were obtained from adult T.molitor by methods previously described (Section 2.3.) and sorted into pools of 1000 individuals in KRT saline. Pre-incubation and post-incubation washing was as described in Section 2.4.3., and incubation for the required time period was in 5ml of the test solution. After washing, parasites were homogenised in 5 ml of 0.4 M perchloric acid in an ice-cold glass homogeniser for 4 min. The homogenate was then decanted into a centrifuge tube, covered with parafilm and allowed to stand for 1h in ice, prior to centrifugation. The supernatent was retained and the pellet was resuspended in 0.4 M perchloric acid and recentrifuged- the entire process being repeated three times. Three perchloric acid washes were found suitable to remove all perchloric-acid soluble ^{14}C-activity from the pellet. The pellet was then dissolved in 2 ml of 1.0 M NaOH overnight and, after neutralisation with HCl, an aliquot was assayed for radioactivity. A similar assay was performed on the supernatents from the three washings.

A washed protein precipitate, derived from 1000 cysticercoids incubated in 0.1 mM ^{14}C-L-leucine for 1 h, was hydrolysed in 0.5 ml 8 M H_2SO_4 for 5 h at 100°C. The hydrolysate was evaporated to dryness, resuspended in 0.2 ml distilled water, spotted onto a silica gel G-coated plate and consequently separated and assayed for radioactivity as described in Section 2.4.6.

6.3. Results

6.3.1. TLC plate separation

All the radioisotope recoverable from the silica gel strips was present in the amino acid spot corresponding with that of authentic L-leucine. This result was found in six replicate experiments, and thus it was concluded that all ^{14}C in protein was in the form of labelled leucine.

6.3.2. ^{14}C-amino acid incorporation into protein

The term 'incorporation' is here used to designate amino acid incorporated into protein and precipitated with perchloric acid. The term 'uptake' represents perchloric acid soluble free-pool material.

Data are expressed as μmoles (mean ± S.E.) amino acid, incorporated or absorbed, per gram ethanol extracted dry weight cysticercoid, per unit time (for mean dry weights of 25 cysticercoids, see Figure 2.4.7.2.1., and multiply the result for each age by 40). Protein incorporation is also expressed as a percentage of the total amino acid absorbed.

The uptake and incorporation of four ^{14}C-L-amino acids are presented in Table 6.3.2.1. Proline was chosen due to the high proportion of collagen within the cysticercoid, and it is

also an example of an imino acid; alanine was chosen as an example of an 'A' site-preferring neutral amino acid, and is also a non-essential amino acid in man and the albino rat (see Section 6.4.); leucine was chosen as an 'L' site-preferring neutral amino acid, and also as an essential amino acid; finally, phenylalanine represents an aromatic amino acid, which may enter adult H.diminuta through a separate uptake locus, and is also an essential amino acid.

The results indicate that there is little correlation between pool size and incorporation rate. The former was broadly similar for all substrates but the percentage incorporation rates varied widely in the order leucine>phenylalanine>alanine>proline. The results obtained by Harris & Read (1969) with the adult worm are provided for comparison in Table 6.3.2.2.

Since the highest rate of incorporation was obtained with leucine, and TLC studies had revealed it to be relatively metabolically stable, this amino acid was selected for further studies.

Figure 6.3.2.1. depicts the incorporation and uptake of ^{14}C-L-leucine from an initial concentration of 0.1 mM over a 5 h time period. Uptake is non-linear but a linear relationship between incorporation and time is apparent ($r = 0.98$; $p = 0.05$ with three degrees of freedom). It is recognised, however, that, despite the good linear fit, a biphasic pattern of incorporation may obtain, as described for glycine incorporation in S.mansoni

Table 6.3.2.1. Uptake and incorporation rates of ^{14}C-amino acids

Amino acid (0.1 mM)	n	Uptake	Incorporation	%Incorporation
leucine	10	6.88 ± 0.77	0.65 ± 0.07	8.6
alanine	4	8.38 ± 1.55	0.19 ± 0.03*	2.2
phenylalanine	4	6.01 ± 0.30	0.36 ± 0.06*	5.6
proline	4	4.26 ± 0.15	0.03 ± 0.002*	0.7

All values obtained with pools of 1000 cysticercoids aged 12-20, incubated for 1 h at 26°C. * designates result significantly different from that of leucine at p = 0.05.

Table 6.3.2.2. Uptake and incorporation rates of ^{14}C-amino acids

Amino acid (1.0 mM)	n	Uptake	Incorporation	%Incorporation
methionine	8	38.5 ± 0.95	0.46 ± 0.15	1.2
leucine	8	35.2 ± 1.06	1.41 ± 0.09	3.9
valine	8	52.1 ± 3.13	1.13 ± 0.06	2.1
lysine	8	9.9 ± 0.04	0.94 ± 0.03	8.7

All values obtained with 10-day -old adult H.diminuta. All values obtained after 1 h incubation at 37°C (data from Harris & Read, 1969).

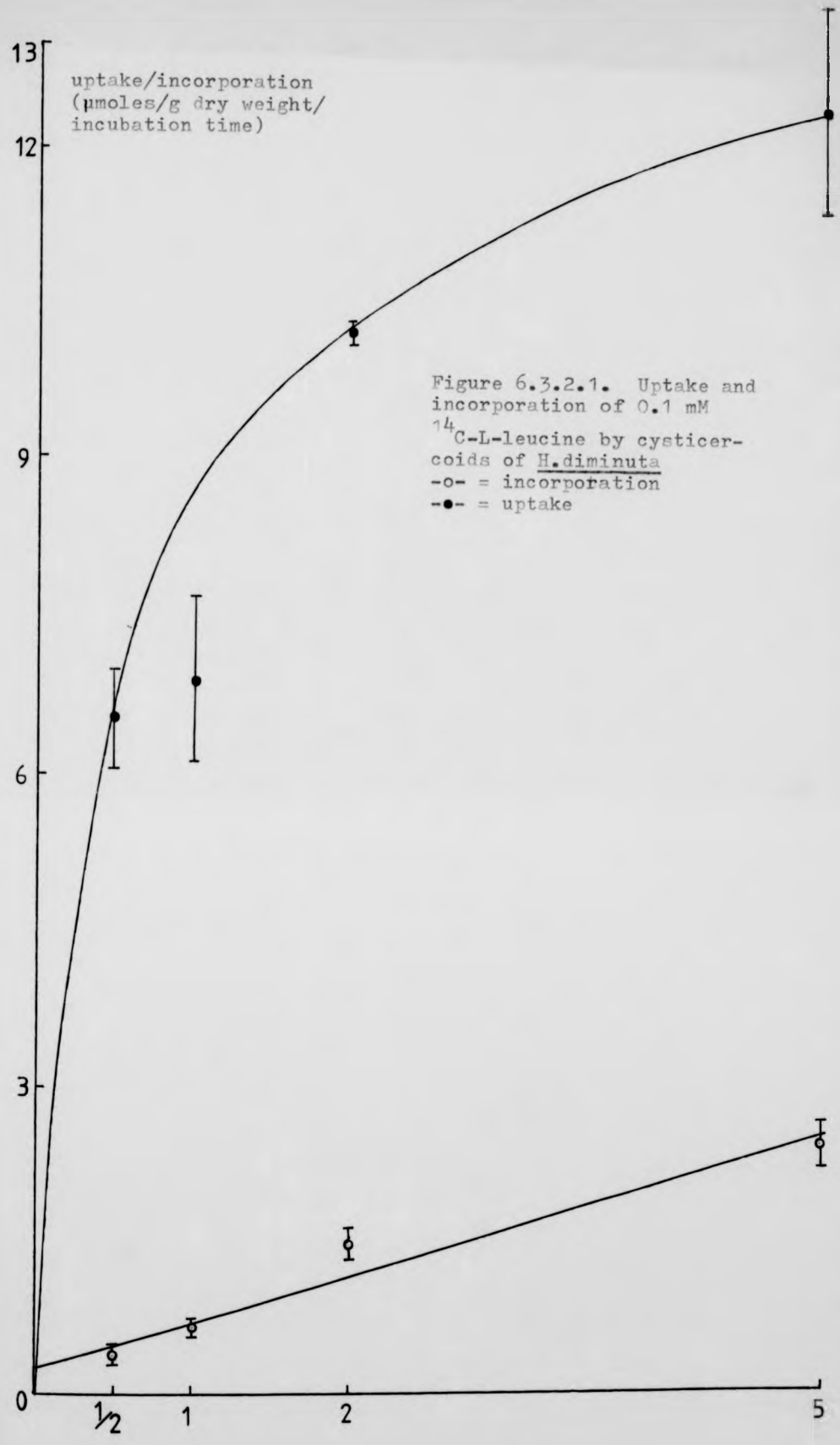


Figure 6.3.2.1. Uptake and incorporation of 0.1 mM ^{14}C-L-leucine by cysticercoids of H.diminuta
-o- = incorporation
-●- = uptake

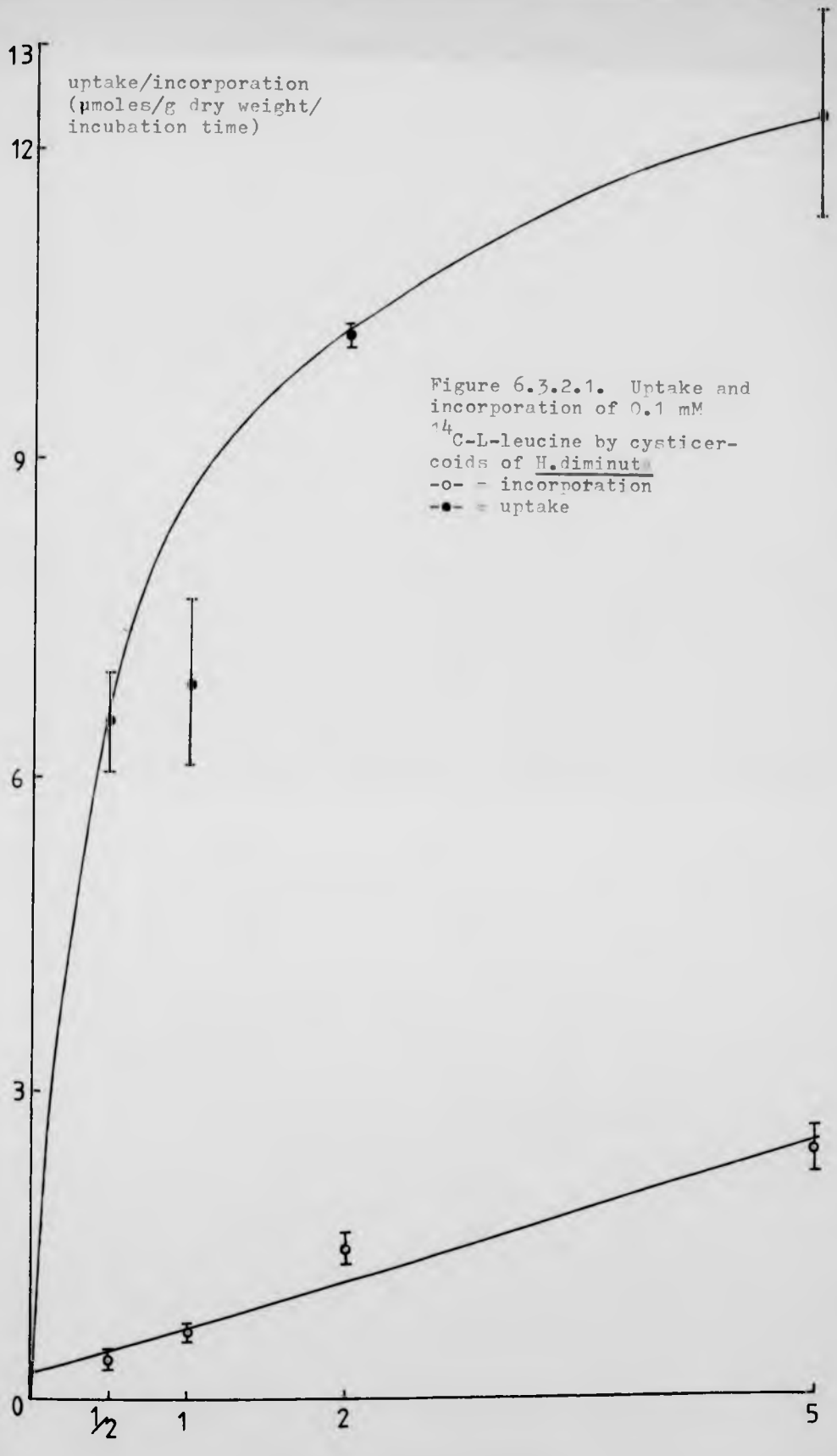
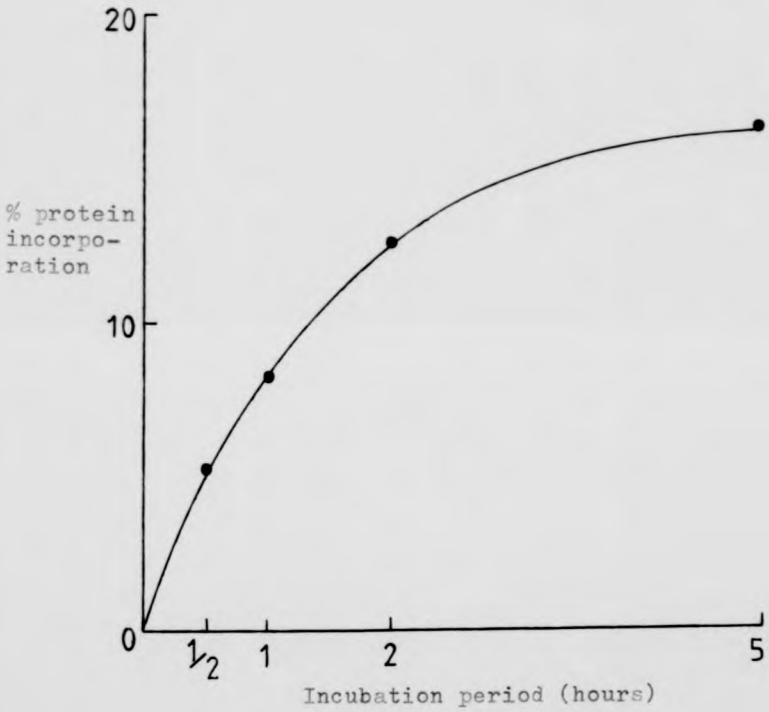


Figure 6.3.2.1. Uptake and incorporation of 0.1 mM ^{14}C-L-leucine by cysticercoids of H.diminuta
-o- = incorporation
-●- = uptake

Figure 6.3.2.2. Mean % incorporation of 0.1 mM ^{14}C-L-leucine by cysticercoids of H.diminuta over increasing incubation periods



by Walker & Chappell (1982). However, when the relationship between percentage incorporation and time is examined a linear relationship is not apparent (Figure 6.3.2.2.).

The dependence of incorporation rates upon initial medium concentration is presented in Table 6.3.3.1. Values obtained with the adult worms are again provided for comparison.

6.3.3. Age-dependent changes in the uptake and incorporation rates of ^{14}C-L-leucine

Figure 4.5.2.4. suggests that the values of V_{max} (cycloleucine) declines as the cysticercoid ages, but that the value of K_t remains constant with increasing age (Figure 4.5.2.3.). Table 6.3.3.1. shows the uptake and incorporation rates obtained with stage III and stage V cysticercoids after 1 h incubation in either 0.1 or 1.0 mM ^{14}C-L-leucine. Similar results obtained with the adult worm by Harris & Read (1969) are provided for comparison. The results indicate that the uptake and incorporation rates of stage III cysticercoids exceed those of both stage V cysticercoids and the adult worm. When only incorporation rates are considered, the rate from 1.0 mM ^{14}C-L-leucine is similar in both the stage V cysticercoids and the adult worm, but almost double these values in the stage III cysticercoid. The change in the percentage incorporation rate as the medium concentration increases by a factor of 10 is a fall to approximately $\frac{1}{2}$ or $\frac{1}{3}$ of the rate obtainable at the lower concentrations in all

Table 6.3.3.1. Uptake and incorporation of ^{14}C-labelled amino acids by stage III cysticercoids, stage V cysticercoids and adult H.diminuta.

^{14}C-amino acid	[S] mM	n	Uptake	Incorporation	% Incorporation
a.) Stage III cysticercoids					
leucine	0.1	4	15.29 ± 3.49	1.48 ± 0.14	8.8
"	1.0	2	139.76 ± 12.55	3.62 ± 0.36	2.5
b.) Stage V cysticercoids					
leucine	0.1	10	6.88 ± 0.78	0.65 ± 0.07	8.6
"	1.0	5	57.25 ± 1.30	1.25 ± 0.12	2.2
c.) Adult worms (data from Harris & Read, 1969)					
lysine	0.1	8	4.53 ± 0.14	0.88 ± 0.04	16.3
"	1.0	8	9.94 ± 0.34	0.94 ± 0.01	8.6
methionine	0.1	8	8.18 ± 0.42	0.41 ± 0.02	4.8
"	1.0	8	31.34 ± 2.02	0.48 ± 0.02	1.5
leucine	1.0	8	35.20 ± 1.06	1.41 ± 0.09	3.9

three examples. In the adult worm, the actual amount of protein synthesised does not fall as the medium concentration is raised, but a contrary result is noted with both cysticercoid stages.

6.3.4. Effects of sugars and inhibitors of protein synthesis

Table 6.3.4.1. presents the effects of the inhibitors puromycin and cycloheximide, as well as glucose, 2-deoxy-D-glucose (2-DOG, a non-metabolisable sugar) and trehalose (a disaccharide present in Tenebrio haemolymph) on the uptake and incorporation rates of ^{14}C-L-leucine. All incubations were for 1 h at 26°C, and the pre-incubation medium also contained the test compound at the same concentration as that used in the incubation medium. The results suggest that the only compound to have any effect on the rate of incorporation was cycloheximide, but that 2-DOG and 25 µM cycloheximide both had an effect on the rate of uptake.

Table 6.3.4.1. Effects of various compounds on the uptake and incorporation of 0.1 mM ^{14}C-L-leucine by stage V cysticercoids

Addition	n	Uptake	Incorporation	%Incorporation
None	10	6.88 ± 0.77	0.65 ± 0.07	8.6
0.1 mM puromycin	3	5.40 ± 0.65	0.57 ± 0.05	9.6
25 µM cyclohex-imide	3	3.51 ± 0.43*	0.16 ± 0.02*	4.4
50 µM "	4	4.45 ± 0.71	0.14 ± 0.03*	3.1
100 µM "	4	4.34 ± 0.39	0.14 ± 0.01*	3.1
0.1 mM glucose	4	8.99 ± 1.46	0.67 ± 0.10	6.9
0.1 mM 2-DOG	4	9.82 ± 0.47*	0.47 ± 0.02	4.6
0.1 mM trehalose	4	4.45 ± 0.43	0.63 ± 0.07	12.4

* denotes result significantly different (@ p = 0.05) from control (no addition).

6.4. Discussion

Protein incorporation *in vitro* from previously absorbed amino acids has been demonstrated in a variety of parasitic helminths (see Table 6.4.1.). An examination of Table 6.4.2. reveals that the degree of incorporation apparently varies with the individual amino acid. Despite the variety of techniques and incubation times employed in the studies referred to in Table 6.4.2., it is noteworthy that the essential amino acids of man and the albino rat (viz. arg,his, lys, leu, ile, phe, met, thr, trp, val) are more readily incorporated into protein than the non-essential amino acids (gly, pro, ala, cys, glu, asp, asn, gln, ser, tyr). The essential amino acids of parasitic helminths are not known, although a few free-living nematodes have been found to require tyrosine in addition to the essential amino acids of man for normal growth (Brockelman & Jackson, 1978).

Very little is known of the ability of helminths to synthesise and interconvert amino acids. *H.diminuta* is capable of synthesising glycine from serine and *vice-versa*, and can carry out a variety of transamination reactions leading to the formation of alanine, aspartate and L-glutamate (see Barrett, 1981, for further details).

When the time course of amino acid incorporation into protein is considered, a linear relationship, similar to that shown in Figure 6.3.2.1., is generally found. The incorporation of ^{14}C-valine into *Calliobothrium verticillatum* protein, ^{14}C-lysine

Table 6.4.1. *In vitro* protein incorporation studies concerning parasitic helminths

Species	Amino acid incorporated	Reference
Ascaris lumbricoides	gly	Harpur & Jackson, 1975
Dirofilaria immitis (microfilariae)	glu, gly, leu, lys, phe	Jaffe & Doremus, 1970
Mermis nigrescens (larvae)	leu, trp, met, arg, gly, glu	Gordon & Webster, 1972
Nippostrongylus brasiliensis (larvae)	pro	Bonner, Weinstein & Saz, 1971
Trichinella spiralis (larvae)	gly, ala (1); tyr, trp (2)	1) Hankes & Stoner, 1956; 2) Stoner & Hankes, 1958
Echinococcus granulosus (hydatid cyst)	mixtures	Agosin & Repetto, 1967
H.diminuta (adult)	met, leu, val, lys (1); lys (2)	1) Harris & Read, 1969 2) Chappell & Read, 1973
Taenia crassiceps (larvae)	leu	Culbreth, Esch & Kuhn, 1972
Fasciola hepatica	tyr, met, leu, phe	Hanna, 1975
Fasciolopsis buski	leu	Cain, 1969
Philophthalamus megalurus	leu	Cain, 1969
Schistosoma mansoni	leu, val (1); leu (2); gly (3)	1) Nagai et al., 1977 2) Walker & Chappell, 1980 3) Walker & Chappell, 1982

Table 6.4.2. Comparison of percentage incorporation rates (in brackets) of amino acids into proteins

D.immitis (1)	M.nigrescens (2)	H.diminuta (3) (adult)	H.diminuta (4) (adult)
gly (9.6)	glu (0.4)	met (1.2)	glu (6)
glu (9.8)	gly (2.2)	val (2.1)	ala (8)
leu (22.1)	arg (14.8)	leu (3.9)	pro (15)
phe (22.9)	met (15.9)	lys (8.7)	gly (16)
lys (23.0)	trp (20.8)		leu (38)
	leu (35.2)		lys (44)
			arg (50)
			val (52)
			phe (55)

H.diminuta (5) (cysticercoid)
pro (0.6)
ala (2.2)
phe (5.6)
leu (8.6)

References

1) Doremus & Jaffe (1970)
2) Gordon & Webster (1972)
3) Harris & Read (1969)
4) MacInnis et al. (1976)
5) This study - see Table 6.3.2.1.

Table 6.4.3. Differing sensitivities to cycloheximide and puromycin

Experimental material	Drug	Maximum % inhibition	Reference
CYCLOHEXIMIDE			
S.mansoni (adult)	3.6×10^{-3}M	75	Walker & Chappell (1980)
" (schistosomula)	3.6×10^{-4}M	0	Nagai et al. (1977)
Tetrahymena pyriformis	7×10^{-7}M	90	Walker & Wheatley (1979)
HeLa cells	1.8×10^{-5}M	80-90	Wheatley & Henderson (1974)
PUROMYCIN			
H.diminuta (adult)	4.6×10^{-5}M	77	Parker & MacInnis (1977)
H.diminuta (cyst)	1×10^{-4}M	0	This study
S.mansoni (adult)	9.2×10^{-4}M	71	Nagai et al. (1977)
Rat liver cells	2.0×10^{-7}M	99	Yarmolinsky et al. (1959)

into H.diminuta protein and ^{3}H-leucine into S.mansoni protein all follow this pattern (Fisher & Read, 1971; Harris & Read, 1969; Walker & Chappell, 1980). However, the incorporation of ^{14}C-leucine into the parasitic larvae of the mermithid nematode Mermis nigrescens is not strictly linear over a 1 h period and the incorporation of glycine into S.mansoni protein follows a biphasic pattern (Walker & Chappell, 1982). Such linear incorporation might suggest a controlled process occuring at a controlled rate. This rate varies for different amino acids but is apparently influenced by the size of the amino acid pool and also by medium amino acid concentration. The studies of Harris & Read (1969) with adult H.diminuta suggest that once a critical pool amino acid concentration has been attained the rate of protein production will remain constant irrespective of pool size. This is consistent with the view that there is a definite basal rate at which protein synthesis can occur and that this rate is controlled at the level of amino acid activation rather than pool concentration, at least above the critical level mentioned above (Chappell & Read, 1973). Relevant discussion of the relationship between amino acid pool formation and the rate of protein synthesis in mammalian systems may be found in Wheatley & Robertson (1981).

It is noteworthy that protein synthesis occurs at a faster rate in the stage III cysticercoid than either the stage V cysticercoid or adult worm. This may be due to an increased number of ribosomes per unit mass available to this developmental stage or an increase in the rate of synthesis. There is no

evidence either for or against the first hypothesis, but it is conceivable that an enhanced rate of synthesis could be due to the utilisation of energy sources unavailable to, or restricted in, later stages of the life cycle. From the available evidence, it would appear that tapeworms possess a 'classical' metabolism, with the catabolism of carbohydrates driving an anabolic protein metabolism (Barrett, 1981). Two examples of this are the enhanced rate of protein synthesis by H.diminuta and Ascaris intestine in the presence of glucose (Harris & Read, 1969; Harpur & Jackson, 1975). Table 6.3.4.1. suggests that glucose has no effect on the rate of protein synthesis by stage V cysticercoids, but it must be noted that such an effect was only noted in the adult stage when the worms were derived from previously starved hosts. A previous study had determined that starving the host for 20 hours depleted the polysaccharide reserves of the worm (Read, 1956) and it was reasoned that the rate of protein synthesis would also fall in the absence of a suitable energy source. No data are available concerning the effects of host starvation on the metacestode stage of the life cycle. A possible alternative energy source could be trehalose, which Barrett (1981) has suggested could serve as a tissue specific metabolite, and the enzyme responsible for its hydrolysis to glucose, trehalase, is known to occur in certain tissues of Ascaris (Lapp & Mason, 1978). Again, however, this compound has no effects on the protein synthetic rates of the stage V cysticercoid. No explanation can be offered to explain the effects of 2-DOG on the rate of uptake of ^{14}C-L-leucine. A third possibility is a non-carbohydrate energy source. It is

known that the rate of ^{14}C-leucine incorporation by the schistosomula of S.mansoni can be enhanced by the addition of 10 non-essential amino acids to the incubation medium (Nagai, Gazzinelli, De Moraes & Pellegrino, 1977). This is at a stage of development when permeability to amino acids has been newly established (Ramalho-Pinto, Gazzinelli, Howells, Mota-Santos, Figueiredo & Pellegrino, 1974). Further work is obviously required in this area.

The inhibitory effects of cycloheximide and puromycin on a number of organisms is presented in Table 6.4.3. All of the parasites involved were used as whole animals, except for the study of Parker & MacInnis (1977) who used a cell-free ribosomal fraction of H.diminuta. Puromycin is believed to act as an analogue of the acylated t-RNA molecule and prevents elongation of the peptide chain on the ribosome. The compound effectively inhibits protein synthesis by both rat liver and H.diminuta ribosomes (Yarmolinsky & De la Haba, 1959; Parker & MacInnis, 1977). Cycloheximide blocks peptide bond formation and binds specifically to the 80s ribosomes of eukaryotic cells (Lehninger, 1975). Table 6.3.4.1. reveals that maximal inhibition of protein synthesis by H.diminuta cysticercoids occurs at a cycloheximide concentration of 2.5×10^{-5} M. A similar degree of inhibition at this concentration is also apparent in HeLa cells. Suprisingly, cycloheximide also affects the uptake of ^{14}C-L-leucine by cysticercoids. A possible explanation for this phenomena may come from the study of Jeffers (1979), who found that cycloheximide disrupted the structure of

the reproductive organs of Microphallus similis. If this compound has similar effects on the absorptive tegument of the cysticercoid, normal absorption could well be impaired. Suprisingly, the cysticercoids were insensitive to puromycin. Possible explanations for this include permeability barriers to the drug, drug inactivation, or too low a concentration of the drug to have any effect. However, a more likely explanation is that the various stages of the life cycle differ in their sensitivities to drugs. Such differences have been noted between the schistosomula and adult stages of S.mansoni (Coles, 1973).

When the role of protein synthesis in parasitic helminths is considered, one finds few reports on the synthesis of specific proteins or the role of proteins in growth. However, interest is growing in the rate of protein turnover as a measurement of the replacement rates of tegument brush border membrane components. With a few exceptions (see Section 1) cestodes cannot absorb proteins from their surroundings, and therefore all of their protein requirements must come from previously absorbed amino acids. In the adult H.diminuta, protein synthesis appears to take place in the tegument-perikarya (Oaks & Lumsden, 1971), and in the stage V cysticercoid is most active in the tegument of the capsule and the body/tail junction (Prescott & Voge, 1959). There are few reports on the synthesis of specific proteins. Cain (1969) was able to show the partial synthesis of haem by Philophthalamus megalurus and Fasciolopsis buski and Bonner, Weinstein & Saz (1971) the synthesis of collagen from previously

absorbed ^{14}C-proline by Nippostrongylus brasiliensis. No evidence is available concerning the role of proteins in the growth of cestodes, although the careful study of Gordon & Webster (1972) has shown the close relationship between the rates of protein synthesis and the rate of weight increase in the growth and development of larval M.nigrescens.

The rate of production, and consequent secretion, of tegument brush border membrane components by helminths has been investigated by a number of workers. Oaks & Lumsden (1971) have found that the surface glycocalyx of both H.diminuta and Lacistorynchus tenuis is replaced every 6-8 hours. Shannon & Bogitsh (1971) suggest a period of 5 hours for a radiolabelled protein to pass from the tegument cells to the glycocalyx of the digenean Megalodiscus temperatus. The replacement of S.mansoni tegument membrane appears to consist of two phases. A rapid secretion of the glycocalyx with a half-life of 2-3 hours (Wilson & Barnes, 1977) and a slower replacement of the plasmamembrane which takes 24-42 hours (Kusel, Sher, Perez, Clegg & Smithers, 1975). It has been proposed that this rapid rate of turnover may be part of the parasite's resistance to the hosts immune attack. With an apparent absence of any host resistance to the presence of the metacestode stage of H.diminuta in a wide variety of arthropods (Section 2.4.7.), it would be of great interest to see how the rate of membrane turnover in this stage of the life cycle compares with that of the adult worm.

To conclude, protein synthesis by the cysticercoid of *H.diminuta* resembles other helminths in its pattern of amino acid incorporation, linear relationship between time and incorporation, non-relationship between amino acid pool size and incorporation, inverse relationship between larval biomass and amino acid incorporation and sensitivity to cycloheximide. It does not resemble the adult worm in its apparent insensitivity to carbohydrates and puromycin. So little is known of the biochemistry and physiology of the cysticercoid that most comparisons with other helminths are mere speculation, but a number of interesting lines of investigation that could be followed have been pointed out.

7. GENERAL DISCUSSION

The results presented in this study have shown that the amino acids cycloleucine, α-AIB, L-alanine, L-proline, L-leucine and L-phenylalanine are absorbed by mediated transport mechanisms and accumulated within the cysticercoid of H.diminuta. At least three loci serve for the transport of these six compounds, and these have characteristics similar to equivalent loci in the adult worm. The purpose of this discussion is to review the nutritional adartions of the cysticercoid to the parasitic mode of life, but it must be stated from the outset that our knowledge of cysticercoid biology is almost non-existent and thus this review, of necessity, draws upon pertinent information relating to the adult H.diminuta. However, one cannot assume that aspects of the biology of the adult worm are the same as those of the cysticercoid and therefore caution is necessary in comparing results.

From the available literature, it would appear that a combination of behavioural, physiological and morphological adaptions allow adult H.diminuta to compete successfully for the available nutrients with its rat host. Adult H.diminuta are known to undergo diel migrations up and down the small intestine of the rat host, probably in response to the feeding patterns of the host (Read & Kilejian, 1969; Arai, 1970). Shortly after the rat has fed, the bulk of the worm population is found in the anterior half of the small intestine, presumably to take advantage of the available glucose which is at its

highest concentration at this time, and also because the vast majority of this compound is absorbed in the anterior parts of the rat gut. This migration, however, does not appear to occur in response to a concentration gradient of glucose as the worm appears to be unable to detect such gradients (Mettrick, 1972). Recent work has shown that the migratory activity is correlated to gradients of 5-hydroxytryptamine, a compound secreted from rat gut cells upon feeding, and which stimulates muscle response in H.diminuta (Mettrick & Cho, 1981a, b). Hopkins & Allen (1979) have shown that the worm detects and maintains its position in the gut of the host. When the worm is first introduced into the gut it actively selects a region approximately 30-50% down the intestine (Bruten & Hopkins, 1969). Hopkins & Allen have shown that, although the stimuli which the worms receive as location cues are unknown, they must exist, and the worm, by monitoring information about its position from all over its strobila, maintains its position as it grows by balancing the input of adverse information from its "tail" and "head" ends. The work of Mettrick & Cho suggests that the location stimuli may well be related to the gradient of 5-hydroxytryptamine in the rat gut, although further work is needed to clarify this.

The development of adult H.diminuta is highly dependent on the quantity and quality of carbohydrate (Read & Simmons, 1963; Mettrick & Munro, 1965) and a shortage of this resource causes worms from above a particular population threshold to become stunted - the so-called 'crowding effect' (Roberts, 1966). An examination of the distribution of microtriches along the

strobila of H.diminuta reveals that they are most numerous at the posterior end of the worm (Rothman, 1963; Mettrick & Cannon, 1970). This may be a modification to ensure that the amounts of nutrients received by the worm are equal along the length of the strobila. An unpublished study reported in Roberts (1980) has shown that an equal amount of glucose is absorbed along the length of the strobila of H.diminuta.

The biological properties of the interface (see Section 1) may also be of crucial importance to the biology of the worm. As stated in Section 1., the brush border membrane is overlain by a glycocalyx which bears a net negative charge. As a result, cations are attracted and the structure may function as a 'cation exchange resin' (Lumsden, 1972). The importance of this is that many of the brush border membrane-bound enzymes possessed by the worm and the enzymes of host origin that are adsorbed onto the glycocalyx (reviewed by Pappas, 1983a, b) show enhanced activity in the presence of certain divalent cations. In addition, the glycocalyx may prevent the diffusion away from the brush border membrane-located transport loci of the breakdown products of enzymic reactions. However, the glycocalyx and its associated proteins may also act as a rate limiting step to the transport of solutes from the intestinal lumen to the transport loci. This could be the reason for the 'stationary water layer effects' frequently referred to by Podesta and Mettrick. Returning to enzymes, several of these have been found associated with the brush border membrane of adult H.diminuta (see review by Pappas, 1983b) and clearly

enhance the supply of nutrients available to the worm. For example, adult H.diminuta is impermeable to phosphorylated monosaccharides, nucleotides or glycerophosphates, but the products of their hydrolysis are readily absorbed (Dike & Read, 1971; Pappas & Read, 1974; Kuo, 1979; Uglem, Pappas & Read, 1974). In certain cases, enzymes and transport loci are in such close proximity to each other on the brush border membrane that the products of hydrolysis do not diffuse away from the surface of the worm but are rapidly absorbed. This confers a 'kinetic advantage' on the worm (Read, 1971).

Other factors also allow the worm to compete successfully for the available nutrients. Firstly, the adoption of carrier-mediated transport systems will serve to enhance the absorption of compounds which are present at low concentrations in the ambient medium. The affinity of these carriers for certain compounds is often similar to the concentrations of those compounds in the medium. For example, K_t(glucose) for H.diminuta varies between 0.74 and 1.6mM (depending on which authority one takes; see Table XI, Pappas & Read, 1975), while the concentration of this compound in the rat gut is 1.5-12mM (Symons- in Fairbairn, 1970). In addition, the affinities of the transport systems possessed by adult H.diminuta may exceed those of the host. MacInnis et al. (1976) have established that this is the case for the amino acid porters of adult H.diminuta and the rat gut. Secondly, the existence of stationary water layers gives the tapeworm an absorptive advantage over the rat gut. If the effects of stationary water layers are not taken into

account, the rat gut would have twelve times more absorptive capacity per unit weight than the adult H.diminuta (Wilson & Dietschy, 1974). But the presence of these layers reduces the potential absorptive capacity of the rat gut to that provided by the tips of the microvilli. The tapeworm also suffers a drop in absorptive capacity but overall it maintains a 3-4 times greater absorptive surface than the rat (Befus & Podesta, 1976). Comparative measurements of the rate of water, electrolyte and non-electrolyte transport by the parasitised rat gut and adult H.diminuta show that the capacity of the tapeworm to absorb these compounds is equal to, or even exceeds that of the host (Podesta & Mettrick, 1976).

When the relationships between the tapeworm and the available amino acids are considered it would appear that the worm is in a state of dynamic equilibrium with the surrounding medium. Read et al. (1963) provided evidence that the influx and efflux of amino acids into and out of adult H.diminuta was heavily influenced by the composition of the surrounding medium and Chappell & Read (1973) have confirmed that the composition of the internal amino acid pools of the worm reflect the composition of the ambient medium. At the time of Read et al.'s publication, the molar ratios of amino acids within the intestines of the dog (Nasset, 1957), smooth dogfish (Read, Simmons, Campbell & Rothman, 1960) and rat (Nasset & Ju, 1961) were thought to remain constant irrespective of diet, as the continual endogenous production of amino acids (through gut secretions, sloughed epithelia etc.) was considered to be of sufficient magnitude to swamp any

transitory, diet-induced changes in the amino acid composition of the gut. Read used this idea to suggest that the parasitic relationship between the rat and H.diminuta went beyond the mere competition for available nutrients, and suggested that the tapeworm was able to take advantage of the constant ratios of amino acids provided for it by the homeostatic mechanisms of the host to regulate its own growth and development. Hopkins & Cullow (1965) supported this theory, and consequent work has centered around attempts to prove that the homeostatic control of gut contents can be overridden by feeding the host large doses of specific amino acids, which would presumably alter the composition of the tapeworm's internal amino acid pool thus affecting its rate of protein synthesis and retarding its growth. Hopkins (1969) and Chappell & Read (1973) have shown that the homeostatic control of rat gut contents can be overcome by feeding the host large doses of methionine or proline respectively. Furthermore, the thorough study performed by Mettrick (1970) has shown that following a meal of casein or albumin, the composition and molar ratios of amino acids within the gut of the rat varied, and that the degree of variation was dependent upon the portion of the gut examined and the time allowed to elapse after feeding. Mettrick concluded that endogenous protein was insufficient to maintain constant molar ratios of amino acids within the gut, thus rejecting Read's theory. However, the actual effects of such an imbalance upon worm growth are still unclear. Chappell & Read (1973) found that an imbalance in the host gut amino acid composition had no effect on the rate of ^{14}C-L-lysine incorporation into protein by adult

H.diminuta. Hopkins & Young (1967) found that host gut pool imbalance had little effect on the rate of growth of adult H.diminuta, but both Mettrick & Munro (1965) and Mettrick (1968) noted the contrary result. Such a diversity of results may result from the migratory nature of the worm, amino acid toxicity or the differing diets employed (Arme, 1975). However, a recent theory put forward by Wheatley (1980) and backed with some experimental evidence (Wheatley & Robertson, 1981) suggests that as the amino acid transport systems of many cells cannot control the composition of their internal pools, the cell lets in 'all comers', including amino acid analogues, and then selects its requirements for protein synthesis from the pools so formed. Some of these analogues are also incorporated into protein (Wheatley, 1980). Thus it would appear that, in some cells at least, the control of protein synthesis does not occur at the transport level. At the present time, the exact amino acid requirements for the normal growth of hymenolepid tapeworms are not known. The oncosphere of H.nana has been successfully cultivated from oncosphere to cysticercoid (Berntzen, 1970) and the cysticercoids of H.diminuta, H.nana, H.microstoma and H.citelli to the mature adult (Berntzen, 1961; Voge, 1975; Berntzen, 1962; DeRycke & Berntzen, 1967; Voge & Green, 1975) but all of the culture media used contained either every amino acid or were undefined.

In contrast, nothing is known of the adaptions, if any, of the cysticercoid to the parasitic mode of life. Although it possesses muscles (at least for the first two weeks of

development (Richards & Arme, 1984)) and surface sensory structures (Ubelaker, 1980) which could enable the cysticercoid to orientate itself to nutritionally favourable sites within the insect host, it is not actually known whether it does so. Observations during the course of this study suggest that the Stage III, IV and V cysticercoid never move *in vitro*, apart from the occasional twitch of the 'tail'. Circumstantial evidence for the existence of such favoured sites within the insect host comes from the observations of McDonald & Wilson (1964) who found that low densities of *H.diminuta* cysticercoids (less than 18) were found in the mesometathorax of *T.confusum*, whereas higher densities (18+) were located within the abdomen. 'Privileged sites' do exist within insects, but are defined as areas where parasites may escape the haemocytic encapsulation reaction of the host (Lackie, 1976). Such a response is not mounted against *H.diminuta* cysticercoids within *T.confusum* or *T.molitor* (Lackie, 1976). Examples of privileged sites are the development of *Tetria* cysticercoids within a sheath of host serosal membrane in various damsel fly nymphs, the development of certain parasitoids within the nerve ganglia of their host, and the temporary development of *H.diminuta* cysticercoids (up to Stage III or IV) in the spaces between the muscles of the thorax and legs of *P.americana* where h emocytes cannot circulate freely (Rees, 1973; Salt, 1968; Lackie, 1976).

Like the adult worm, the cysticercoid of *H.diminuta* appears to obtain many of its nutrients by mediated transport. Previous studies have shown that α-AIB (Arme & Coates, 1973),

glucose and sodium acetate (Arme et al., 1973) and galactose (Phillips & Arme, 1983) are absorbed by mediated systems, and to this list can be added cycloleucine, L-alanine, L-proline, L-leucine and L-phenylalanine (this study). Table 7.1. compares the affinities of the systems which transport the aforementioned amino acids with the concentrations of these compounds in the haemolymph of adult Tenebrio. Such an analysis suggests that the cysticercoid porters have sufficiently high affinities to take advantage of the available levels of these compounds. Unfortunately, very little is known of the insect porters with which they must compete. Although Schistocerca gregaria and P.americana are known to absorb sugars and amino acids through their mid-guts by diffusion (Treherne, 1957, 1958a, b, 1959) and various lepidopteran larvae have mediated transport systems for the absorption of certain amino acids in the same place (Sacchi, Cattaneo, Carpentieri & Giordanna, 1981), it is not known whether porters exist on the tissues and organs of the insect that are bathed by haemolymph, and thus would be in direct competition with those possessed by the cysticercoid. There is some evidence, however, that storage proteins are absorbed and accumulated by the fat body of Bombyx mori (Tojo, Kiguchi & Kimura, 1981), and vitellogenic proteins are absorbed by the oocytes of Diploptera punctata (Mundall, Tobe & Stay, 1981) and Rhodnius prolixus (Davey, 1981). All of these processes are under hormonal control, and in the case of R.prolixus the proteins are acquired by micropinocytosis (Patchin & Davey, 1968).

The properties of the cysticercoid interface are similarly

ill-defined. The composition of the brush border membrane is not known, and there are no reports of any glycocalyx, adsorbed host proteins or stationary water layers. Some enzymatic activity has been reported in this region, however. Pogitsh (1967) demonstrated the presence of non-specific phosphatase activity in the tegument of H.diminuta cysticercoids, and Moczoń (1973), using histochemical tests, found seven specific or non-specific phosphatases in the same region of the same parasite. The lack of alakaline phosphatase in the tegument of the capsule and the presence of this enzyme in the tegument of the scolex suggested to Moczoń that only the scolex of this parasite was active in absorption. However, Phillips & Arme (1983) have recently shown that both the scolex and the capsule of the cysticercoid of H.diminuta bear mediated transport systems for the uptake of glucose, and that the K_t values describing these processes are different in both locations.

As mentioned in the Introduction (Section 1.), the brush border membrane of adult H.diminuta contains many different loci which serve for the transport of a wide variety of nutrients. Considering general concepts first, the strategy of the tapeworm (and the transporting epithelia of many other animals) is to maintain a small number of loci types with wide specificities, rather than a large number with narrow specificities. The advantage of this strategy is that less genetic capacity is required for the continual synthesis of a small number of loci types than a large number, and studies with bacteria have shown that the bacteria employing this strategy

have an energetic advantage over their competitors (see discussion in Pappas & Read, 1975). A second advantage of the broad specificity loci may be to prevent the flooding of internal tissues by some component of the ambient medium which is present in high concentrations. For example, there is considerable evidence that the rate of uptake of neutral amino acids and their steady-state concentration within the internal tissues of adult H.diminuta is dependent upon the competitive activities of other neutral amino acids at the transport loci (Pappas & Read, 1975). Another strategy that the tapeworm may employ to prevent internal flooding is to lower the affinities of those porters which transport the compound in excess. Proline is by far the most abundant amino acid in Tenebrio haemolymph (Tables 7.2. and 7.3.), and the raised K_t value noted for the transport of this compound by cysticercoids of H.diminuta (compared with the adult value, where proline is not in excess in the ambient medium) is the first piece of experimental evidence to support the existence of such a strategy.

However, despite the broad affinities shown by many tapeworm loci, each loci appears to be specific for a certain group of compounds. Amino acids do not interact with those loci which transport hexose sugars and vice-versa (Section 5.4.). There is some evidence which suggests that certain tapeworm loci are selectively permeable. For example, adult H.diminuta cannot transport fructose via its hexose sugar porters (Arme & Read, 1970). Such an exclusion would be energy-conservative as

as this compound is not metabolised to any significant extent by the tapeworm (Read & Simmons, 1963). Thus, by a combination of broad specificity and specific permeability, the composition of internal pools of metabolites can be regulated, and the desired components extracted from these pools for metabolism.

When the numbers and specificities of the tapeworm porters are compared to those of the rat host, one notes a diversification rather than a simplification (MacInnis et al., 1976). It has been suggested that this may represent a lack of synthetic capacity by the worm (Barrett, 1981) which may explain why the worm possesses two loci for the transport of fatty acids (Arme & Read, 1968; Chappell, Arme & Read, 1969). H.diminuta cannot synthesis these compounds and is thus totally dependent on the host for its supply (Ginger & Fairbairn, 1966; Jacobsen & Fairbairn, 1967), but why this worm has two separate glycerol transport systems, one Na^+-dependent and one Na^+-independent, is open to speculation (Uglem, Pappas & Read, 1974). Both the adult and cysticercoid stages of H.diminuta maintain multiple systems for the transport of neutral amino acids (Figs. 3.3.4. & 5.3.5. respectively) and Pappas & Read (1975) have put forward three theories which attempt to explain this seemingly extravagent provision. By changing the numbers of such multiple systems, the parasite may respond to :-

a.) The environments furnished by more than one host species.

b.) Changes in the physiology of the host associated with age or intercurrent disease.

c.) The presence of other parasites in the host.

There is some circumstantial evidence regarding the third theory. The growth rate of H.diminuta is retarded in the presence of Moniliformis dubius in the gut of the rat host. This is probably a manifestation of the 'crowding effect', possibly resulting from a shortage of nutrients. In addition, H.diminuta is displaced from its normal position in the anterior part of the gut to a position 28-65% further down the gut by M.dubius which takes up residence in the anterior quarter (Holmes, 1961, 1962). Nippostrongylus brasiliensis has also been shown to affect the establishment rate of H.diminuta in the rat gut, but the authors of this report do not consider this to be as a result of competition for nutrients but rather to be a result of the conditions within the gut being altered and rendered unsuitable to the worm by the pathogenic activities of this nematode (Morcock & Roberts, unpublished - see Roberts, 1980).

There is no evidence concerning the second theory. Regarding the first theory, Read et al. (1963) found that when adult H.diminuta were reared in golden hamsters, the worms obtained from these animals after 10 days growth had different uptake characteristics compared to worms reared in rats. In addition to an absolute change in methionine affinity, Read and his co-workers found marked changes in the relative capacities of glycine, hydroxyproline, proline, histidine and phenylalanine to inhibit methionine uptake. They interpreted these results as representing a host-induced change in the

relative numbers of the different loci responsible for the uptake of methionine and associated amino acids. Similar changes in the uptake characteristics of adult H.diminuta also appear to occur as a result of increasing worm mass, age or infection density (see Section 4.6. for discussion).

The biochemical adaptions observable in helminths have been reviewed by Fairbairn (1971). Adaption, in this sense, is defined as a goal-directed condition or state which arises in the individual organism as a direct and necessary consequence of natural selection. Epigenetic adaption is concerned with the orderly growth and development of an organism throughout its life cycle. In helminths this leads to biochemical events appearing, disappearing and even reappearing in precisely programmed sequences in response to the different demands made upon the parasite by its complex life cycle. Helminths also show exploitive adaption, which is the genetically determined capacity of the organism to acclimatise to new situations. This is obviously of importance to helminths with a wide host range, such as the cysticercoid of H.diminuta. The establishment of this parasite in a suitable host is dependent on a number of factors, beginning with the cracking of the egg capsule by suitable insect mouthparts (Lethbridge, 1971a), the activation of the embryo before its release from the remaining envelopes (Ogren, Ogren & Skarnulis, 1969), the enzymatic destruction of the inner envelope and embryophore to facilitate the escape of the oncosphere (Lethbridge, 1971a), the successful penetration of the insect gut wall to enter the haemocoel (Voge & Craiwer,

1964; Lethbridge, 1971a), the evasion of the insects haemocytic encapsulation reaction (Lackie, 1976), and, finally, the possession of suitable nutrient transport systems to obtain the necessary amounts of metabolites for normal growth and development. All of these factors may have an effect on the host specificity of H.diminuta cysticercoids, but for the purposes of this discussion, only the last-mentioned factor need concern us here. Information regarding nutrient-dependent host specificity can be gleaned from in vitro studies with hymenolepid tapeworms. The culture media employed for the in vitro culture of H.diminuta cysticercoids are based upon a medium developed for the maintenance of cockroach cells (Landureau, 1966, 1968). The amino acid composition of this medium, as used for H.citelli (after Voge & Green, 1975; the medium used for H.diminuta is the same except that 1mM cysteine is employed (Voge, 1975)), is shown in Tables 7.2. and 7.3., and compared to the amino acid compositions of T.molitor, P.americana and Locusta migratoria haemolymph and the lumen of the rat gut. Considering only the haemolymphs and the culture medium, it should be apparent that there are considerable differences between the amounts of each amino acid in each solution. H.diminuta cysticercoids are capable of penetrating the gut walls of adult T.molitor and P.americana, but cannot survive beyond Stage III or IV in the latter insect due to its encapsulation response (Lackie, 1976). They are not capable of penetrating the gut walls of the locust, but will develop to maturity if the oncospheres are injected directly into the haemocoel (Lethbridge, 1971c). Thus it would appear

that the environments in which the cysticercoid finds itself (the insect haemocoel or culture medium) are sufficient to supply all its nutritional needs and this would imply that the amino acid transport systems which this parasite possesses are 'flexible' enough to cope with the different molar ratios of these compounds that are present in each medium. This is a good example of exploitive adaption in tapeworms, being due to the genetically-coded flexibilty of the amino acid transport loci.

It is possible that the differences noted between the amino acid transport systems of adult and cysticercoid H.diminuta represent epigenetic adaptions induced by the different physico-chemical environments experienced by these two stages. The 'classic' example of epigenetic adaption in helminths is the radically different metabolisms of the egg and adult of Ascaris lumbricoides (see review by Fairbairn, 1970). Other examples of this phenomena concerning H.diminuta are the distinctly different enzyme kinetics of the lactate dehydrogenases possessed by the cysticercoid and adult worm (Walkey & Fairbairn, 1973), and the presence of two isoenzymes of pyruvate kinase in the cysticercoid compared to five in the adult worm (Carter & Fairbairn, 1975). There have been few other studies of the comparative physiology of larval and adult helminths. Uglem, Pappas & Read (1973) detected differences between the ability of the cystacanth and adult stages of M.dubius to hydrolyse the compound leucylleucine, the hydrolysis products of which caused a marked inhibition of

leucine uptake by the adult stage but not by the cystacanth. Chappell (1974) could detect no differences in the characteristics of the methionine loci of adult, juvenile and schistosomula of S.mansoni, beyond a ten-fold reduction in the amount of methionine absorbed by the schistosomula. Interestingly, the K_t value describing methionine uptake by this worm was 0.33mM, surprisingly close to the value obtained with adult H.diminuta (0.31mM). In contrast, the K_t values describing the uptake of cycloleucine, leucine, alanine, phenylalanine (Isseroff et al., 1976) and proline (Asch & Read, 1975) bear no resemblence to those values calculated for either adult or cysticercoid H.diminuta. The only studies concerning the mediated transport of metabolites by helminths utilising insects, other than those concerned with the cysticercoid of H.diminuta, are those of Rutherford & Webster (1974) and Rutherford, Webster & Barlow (1977) who examined the nutritional physiology of the parasitic juvenile form of the mermithid nematode, H.nigrescens. Mermithid nematodes inhabit the haemocoel of various terrestial and aquatic insects (Jeffs, 1970; Poinar, 1975) and some bear a modified cuticle which is composed of distinct microvilli-like projections (Riding, 1970). However, Larval Mermis does not possess these structures (Webster & Gordon, 1974) and nutrient uptake may occur through pores such as have been detected in a related mermithid, Romanomermis culicivorax (Poinar & Hess, 1976). The two studies with Mermis revealed that the 'cuticle' contained mediated transport systems serving for the uptake of glucose and amino acids. Unfortunately, the adult Mermis is free-living, as are

all mermithids, and possesses a 'normal', impermeable cuticle.

In their 1973 publication, Arme and Coates speculated that "... the similarities in transport systems may reflect a basic similarity in the physico-chemical properties of the insect haemocoel and the rat intestine". This statement would appear to be incorrect when one considers the data of Tables 7.2. and 7.3. At the end of this study, one is left with the impression that, at least with regard to amino acid transport systems, sufficient genetic flexibility has been built into the cysticercoid to enable it to establish in a wide variety of hosts, with certain modifications to cope with extremes, and to make the transfer from one environment to the other with little change. Obviously genetic capacity will be conserved in such a case, and this may go some way to explaining the success of this tapeworm.

Table 7.1. Comparison of T.molitor haemolymph amino acid concentrations with affinities of the equivalent transport loci of Stage V cysticercoids of H.diminuta.

Amino acid	K_t transport loci (mM)	Concentration in haemolymph (mM)
L-pro	0.88	55.9
L-ala	0.30	3.6[b]
L-leu	0.32	1.12
L-phe	0.20	0.59
		(a)

(a) - data from Hurd & Burme (unpublished)

b - also includes citrulline

Table 7.1. Comparison of T.solitor haemolymph amino acid concentrations with affinities of the equivalent transport loci of Stage V cysticercoids of H.diminuta.

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L-phe	0.20	0.59
		(a)

(a) - data from Hurd & Arme (unpublished)

b - also includes citrulline

Amino acid	Medium composition (mg/100ml)				
	T.molitor	L.migratoria	P.americana	H.citelli	rat gut
L-α-ala	32.0	27.6	7.3	12.0	2.93
L-β-ala	-	-	-	4.5	-
L-arg	18.6	16.8	18.9	200.0	2.13
L-asp	-	16.8	2.3	20.0	1.61
L-cys	-	-	-	10.1	-
L-glu	22.1	129.7	23.8	100.0	3.19
L-gln	40.3	-	-	55.9	-
gly	15.7	70.3	52.9	20.0	2.17
L-his	70.1	24.3	23.1	40.4	0.58
L-ile	13.4	24.9	5.6	-	1.34
L-leu	15.6	20.5	6.8	24.9	2.82
L-lys	45.3	39.5	10.9	1.2	2.49
L-met	trace	-	4.2	49.2	0.72
L-phe	9.7	13.5	6.3	-	0.99
L-pro	642.9	113.0	42.5	74.8	1.26
L-ser	11.6	-	14.1	8.3	2.87
L-thr	10.0	13.5	7.6	2.0	1.41
L-tyr	48.9	5.4	25.1	36.2	0.99
L-val	38.4	34.0	10.8	15.2	1.60
Reference	(1)	(2)	(3)	(4)	(5)

(1) Data for haemolymph of non-infected, 15 day-old females (Hurd & Arme, unpublished data)

(2) Data for 5th instar haemolymph plasma (Duchateau & Florkin, 1958)

(3) Data for adult haemolymph (Stevens, 1961)

(4) Data for H.citelli culture medium. L-arg is L-arg HCl, L-his is L-his HCl (Voge & Green, 1975)

(5) Summation of data for eight equal sections of uninfected laboratory rat small intestine (Mettrick, 1971). Data in μmoles/100g body weight

Table 7.2. Amino acid composition of various media in which H.diminuta can establish

Table 7.3. Rankings of various values given in Table 7.2. as percentage of amino acid total quantity (% in brackets)

T.molitor	L.migratoria	P.americana	H.citelli	rat gut
pro (68.0)	pro (21.3)	gly (31.2)	arg (24.1)	glu (11.0)
his (5.5)	gly (20.3)	pro (16.4)	glu (14.3)	α-ala (10.1)
ala (4.4)	glu (19.1)	glu (7.2)	pro (13.6)	ser (9.9)
val (4.0)	ala (6.7)	his (6.6)	gln (8.0)	leu (9.7)
lys (3.8)	val (6.3)	tyr (6.2)	met (6.9)	lys (8.6)
gln (3.4)	lys (5.9)	ser (5.9)	gly (5.6)	gly (7.5)
tyr (3.3)	ile (4.1)	arg (4.8)	his (5.5)	arg (7.3)
gly (2.8)	his (3.4)	val (4.1)	tyr (4.2)	val (5.5)
glu (1.8)	leu	ala (3.6)	leu (4.0)	asp
leu (1.5)	asp (2.7)	lys (3.3)	asp (3.2)	thr (4.9)
ser (1.4)	thr (2.5)	thr (2.8)	α-ala (2.8)	ile (4.6)
arg (1.3)	arg (2.1)	leu (2.3)	val (2.7)	pro (4.3)
ile (1.2)	phe (1.8)	ile (1.9)	cys (1.8)	tyr (3.4)
thr (1.0)	tyr (0.7)	phe (1.7)	ser (1.7)	phe
phe (0.7)	met (0)	met (1.2)	β-ala (1.1)	met (2.5)
met (tr.)		asp (0.8)	thr (0.4)	his (2.0)
			lys (0.2)	

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APPENDIX 1A. Composition of Salines (all quantities in grams)

	Saline		
Compound	1X KR	1X Hanks'	1X Hoyle's
NaCl	7.02	8.00	7.60
$MgSO_4.7H_2O$	0.32	0.10	-
$CaCl_2.6H_2O$	0.57	0.28	0.43
KCl	0.36	0.40	0.75
$Na_2HPO_4.12H_2O$	-	0.12	-
KH_2PO_4	-	0.06	-
$MgCl_2.6H_2O$	-	0.10	0.36
$NaHCO_3$	-	0.35	0.37
$NaH_2PO_4.2H_2O$	-	-	0.48

0.25 M TM buffer: add 500 ml 1 M Tris-maleic acid solution (121.1 g Tris, 116.0 g maleic acid, made up to 1000 ml with distilled water) to 800 ml water, adjust pH to 7.4, bring volume to 2000 ml.

0.1 M phosphate buffer: 35.78 g $Na_2HPO_4.12H_2O$ in water, add 20 ml of 1 M HCl, bring volume to 800 ml with water, adjust pH to 7.4, bring volume up to 1000 ml.

APPENDIX 1B. Balanced electrolyte solution employed by Podest et al. (1977)

ion	mmol/litre	KRT (for comparison)
Na^+	133.0	120.0
K^+	5.0	4.8
Mg^{2+}	1.2	1.2
Ca^{2+}	1.2	2.6
Cl^-	130.0	130.0
HCO_3^-	10.0	(SO_4^{2-}, 1.2; Tris,
HPO_4^{2-}	1.33	25; Maleic acid, 25)
$H_2PO_4^-$	0.67	
mannitol	25.0	

(approximately 310 mosm/kg)

Solution equilibrated with 5% CO_2, 95% N_2, and contained 4g/liter polyethylene glycol 4000

APPENDIX 1C. Hymenolepid culture medium as used by Podesta et al. (1977)

Medium is modified Eagle's Medium supplemented with horse serum and liver extract, buffered with bicarbonate (after Evans, 1970). Ratio is 60ml Eagle's: 30ml horse serum: 10ml liver extract.

Component	Quantity (g/litre)
L-arg HCl	0.042
L-cys	0.024
L-his HCl	0.0192
L-ile	0.0524
L-leu	0.0524
L-lys HCl	0.0731
L-phe	0.033
L-thr	0.0476
L-trp	0.008
L-tyr	0.0362
L-val	0.0468
L-met	0.015
L-gln	0.292
Choline chloride	0.002
folic acid	0.002
nicotinamide	0.002
DL-Ca pantothenate	0.002
pyridoxal HCl	0.002
thiamine HCl (aneurine)	0.002
riboflavin	0.002
inositol	0.0035
NaCl	6.4
KCl	0.4
$CaCl_2$	0.2
$MgSO_4 \cdot 7H_2O$	0.2
$NaH_2PO_4 \cdot 2H_2O$	0.14
$Fe(NO_3)_3 \cdot 9H_2O$	0.0001
$NaHCO_3$	2.75
dextrose	4.5

(cont...

streptomycin sulphate (Glaxo)	0.1
n-butyl-p-hydroxybenzoate	0.0002
penicillin (Glaxo, Crystapen)	100,000 units litre^{-1}
phenol red	0.0015%

The above ingredients are dissolved in distilled water, sterilized by Millipore filtration and stored at 4°C

APPENDIX 1D. Composition of solutions used for electron microscopy

Millonig buffer: 83ml of 2.26% NaH_2PO_4 and 17ml of 2.52% NaOH

4% Millonig buffered gluteraldehyde: 52.5ml of Millonig buffer plus 0.31ml of 1.11% $CaCl_2$. Add 1.9g of sucrose and 1X10ml vial of 25% gluteraldehyde (aqueous TAAB) solution

Millonig's washing buffer: 100ml Millonig's buffer, 0.5ml 1.11% $CaCl_2$, 3.0g sucrose

Double strength Millonig's buffer: 20.75ml of 4.52% NaH_2PO_4, 4.25ml of 5.04% NaOH

1% Millonig buffered osmium tetroxide: 15ml double strength Millonig buffer, 1X5ml vial of osmium tetroxide (aqueous solution), 1 drop of 1.11% $CaCl_2$, 0.6g sucrose

APPENDIX 2. Michaelis-Menten enzyme kinetics (as applied to transport kinetics)

The rate of formation of product (or, in the case of transport, the velocity of transport), v, is given by:-

$$v = k_3[SC] \qquad -(1)$$

at a steady state, the rate of breakdown of SC, governed by the rates k_2 and k_3, will be balanced by its rate of formation, governed by k_1, and thus:-

$$k_2[SC] + k_3[SC] = k_1[C] \cdot [S]$$

$$\therefore [SC] = \frac{k_1}{k_2 + k_3}[C] \cdot [S] \qquad -(2)$$

However, the concentration of (or strictly, numbers of) unbound [C] carriers is not known, but the total number of carriers, C_m, must remain constant and will be equal to the non-bound carriers plus the bound, therefore:-

$$C_m = [C] + [SC] \qquad -(3)$$

From equations 1, 2 and 3, one can eliminate C and SC and relate v to C_TS and the rate constant:-

i) Eliminating C

from (3) $[C] = C_T - [SC]$

$$[SC] = \frac{k_1}{k_2 + k_3}[S] \cdot (C_T - [SC]) \qquad -(4)$$

ii) Substituting for [SC] from (4)

$$[SC] + \frac{k_1 \cdot S}{k_2 + k_3} \cdot [SC] = \frac{k_1 \cdot [S]}{k_2 + k_3} \cdot C_T$$

$$\therefore [SC] = \frac{\dfrac{k_1 \cdot [S] \cdot C_T}{k_2 + k_3}}{1 + \dfrac{k_1}{k_2 + k_3} \cdot [S]}$$

$$= \frac{C_T [S]}{\dfrac{k_2 + k_3}{k_1} + S}$$

iii) Substituting for SC in equation (1)

$$v = k_3 \cdot \frac{C_T [S]}{\dfrac{k_2 + k_3}{k_1} + [S]}$$

Let $k_2 + k_3 / k_1 = K_t$, which is a constant:-

$$v = \frac{k_3 \cdot C_T [S]}{K_t + [S]} \qquad -(5)$$

At high [S] , all the carriers will be bound with substrate to give SC, which breaks down at a rate given by k_3. This is maximum velocity. Therefore (5) can be re-written as:-

$$v = \frac{V_{max} [S]}{K_t + [S]} \qquad -(6)$$

Which is the Michaelis-Menten equation.